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*The Role of Eicosapentaenoic Acid in Cancer
Cachexia*

Sarah Ann Price
Doctor of Philosophy

Aston University
September 1997

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Summary

Cachexia is a wasting syndrome often associated with malignancy, characterised by alterations in host metabolism and significant catabolism of host adipose tissue and skeletal muscle. The MAC16 murine adenocarcinoma is profoundly cachexigenic, inducing host weight-loss at relatively small tumour burden without the induction of anorexia.

A 40kDa factor capable of inducing lipolysis *in vitro* via an activation of adenylate cyclase (AC) has been isolated from the MAC16 tumour, and the urine of cachectic cancer patients, using a series of ion exchange and gel exclusion chromatography procedures. This lipid-mobilising factor (LMF) has been demonstrated to stimulate lipolysis in adipocytes dose-dependently via a signal transduction pathway involving, possibly, β_3 -adrenoceptors.

Oral administration of the n-3 polyunsaturated fatty acid (PUFA) eicosapentaenoic acid (EPA) attenuated the progression of cachexia, but not the production of LMF, in MAC16 tumour-bearing mice, and was significantly incorporated into plasma phospholipids, skeletal muscle and adipose tissue. EPA supplemented cancer patients also demonstrated significantly increased plasma EPA concentrations. Decreased plasma membrane AC activity in response to LMF was observed in adipocytes isolated from mice receiving EPA.

Incubation *in vitro* of adipocytes, or plasma membranes, with PUFAs significantly altered membrane fatty acid composition and attenuated the induction of both lipolysis, and AC activity, by LMF. The inhibitory actions of EPA, but not docosahexaenoic acid, are probably the consequence of an interaction with guanine nucleotide binding proteins (G-proteins). Progression of the cachectic state induced an up-regulation of adipocyte membrane expression of stimulatory G-proteins, allied with a concomitant down-regulation of inhibitory G-proteins, thus facilitating the catabolic actions of LMF, implying some tumour-mediated effect. A reversal of such alterations was observed upon oral administration of EPA, suggesting that the primary mechanism of action of this fatty acid is an inhibition of the end organ effects of LMF.

KEY WORDS: POLYUNSATURATED FATTY ACIDS, LIPID-MOBILISING FACTOR, LIPOLYSIS, ADENYLATE CYCLASE, GUANINE NUCLEOTIDE BINDING PROTEINS

*To Mum and Dad
With Love*

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Abbreviations

AA	Arachidonic acid (all- <i>cis</i> -5.8.11.14-eicosatetraenoic acid)
AC	Adenylate cyclase
ACTH	Adrenocorticotropin hormone
ADP	Adenosine 5'-diphosphate
AIDS	Acquired immune deficiency syndrome
AMP	Adenosine 5'-monophosphate
APPR	Acute phase protein response
APS	Ammonium persulphate
ATP	Adenosine 5'-triphosphate
βARK	β-adrenergic receptor kinase
BAT	Brown adipose tissue
BCAA	Branched chain amino acids
Bq	Becquerels
BSA	Bovine serum albumin
(k)cal	(kilo) calories
cAMP	Cyclic adenosine 3':5'- monophosphate
CCK	Cholecystokinin
CHO	Chinese hamster ovary
Ci	Curies
cm	centimetre
CNS	Central nervous system
CNTF	Ciliary neurotrophic factor
CoA	Coenzyme A
COX	Cyclo-oxygenase
cpm	Counts per minute
CRH	Corticotropin releasing hormone
CRP	C reactive protein

(k)Da	(kilo) Daltons
DHA	All- <i>cis</i> -4,7,10,13,16,19-docosaheptaenoic acid
(c)DNA	(complementary) deoxyribonucleic acid
DPA	All- <i>cis</i> -7,10,13,16,19-docosapentaenoic acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EFA(s)	Essential fatty acid(s)
EGTA	Ethylene glycol-bis(β aminoethylether) N,N,N',N' tetracetic acid
EPA	All- <i>cis</i> -5,8,11,14,17-eicosapentaenoic acid
FADH ₂	Flavin adenine dinucleotide (reduced form)
FFA(s)	Free fatty acid(s)
FPLC	Fast protein liquid chromatography
g	Gram(s)
G-protein	Guanine nucleotide binding protein
GDP	Guanosine 5'-diphosphate
GI	Gastrointestinal
GLA	γ -Linolenic acid (all- <i>cis</i> - 6,9,12-octadecatrienoic acid)
GTP	Guanosine 5'-triphosphate
h	Hour(s)
HPA	Hypothalamic-pituitary-adrenal axis
HPLC	High performance liquid chromatography
HSL	Hormone sensitive lipase
IBMX	3-isobutyl-1-methyl xanthine
IFN- γ	Interferon- γ
Ig	Immunoglobulin
IGF-1	Insulin-like growth factor-1
IL	Interleukin
IL-1RA	Interleukin-1 receptor antagonist
kg	Kilogram(s)

l	Litre(s)
LA	Linoleic acid
LDH	Lactate dehydrogenase
LHA	Lateral hypothalamic areas
LIF	Leukaemia inhibitory factor
LLC	Lewis lung carcinoma
LMF	Lipid-mobilising factor
LOX	Lipoxygenase
LPF	Lipolysis promoting factor
LPL	Lipoprotein lipase
LT	Leukotriene
μ	Micro
M	Moles per litre
m	Milli
MA	Margaric acid
MAb	Monoclonal antibody
MAC	Murine adenocarcinoma of the colon
MCT	Medium chain triglycerides
min	Minute(s)
MLT	Melatonin
NAD	β -Nicotinamide adenine dinucleotide
NADH	α -Nicotinamide adenine dinucleotide - reduced form
NF- κ B	Nuclear factor κ B
NPY	Neuropeptide Y
NSAID	Non-steroidal anti-inflammatory
PAGE	Polyacrylamide gel electrophoresis
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PEP	Phosphoenolpyruvate

PG	Prostaglandin
PIF	Proteolysis-inducing factor
PKA	Protein kinase A (cAMP-dependent protein kinase)
PKC	Protein kinase C
pmoles	Picomoles
PMSF	Phenylmethylsulphonylfluoride
PSW	Pre-illness stable weight
PUFA	Polyunsaturated fatty acid
PVN	Paraventricular nucleus
QS	Q sepharose
REE	Resting energy expenditure
(m)RNA	(messenger) ribonucleic acid
rpm	Revolutions per minute
SCID	Severe combined immune deficiency
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
TAG	Triacylglycerols
TEMED	N,N,N',N'-Tetramethylethylenediamine
TG	Triglyceride
TGF β	Transforming growth factor- β
TNF- α	Tumour necrosis factor- α
TPN	Total parenteral nutrition
Tris	Tris (hydroxymethyl) methylamine
U	Unit(s)
VHN	Ventromedial hypothalamic nuclei
WAT	White adipose tissue
Zn α ₂ gp	Zinc- α ₂ -glycoprotein

Chapter 1 Introduction

1.1 Causes of Death in Cancer Patients

Many and varied complications may be found to be the actual cause of death in cancer patients. In a study into the immediate cause of death in each of 500 differing cases of carcinoma, Warren (1932) documented fatalities due to such disparate complications as pneumonia, pulmonary insufficiency, pulmonary embolus, peritonitis, renal insufficiency, haemorrhage, hepatic insufficiency and intestinal obstruction. Likewise, in a survey of 11,074 cancer patient deaths, Harnet (1952) reported that pulmonary complications, cardiovascular disease, uraemia, peritonitis, haemorrhage, cerebral metastasis or inflammatory complications, surgical shock, intestinal obstruction, sepsis and pulmonary embolism could all be implicated. However, by far the most prevalent cause of death associated with both patient groups was the progressive syndrome of wasting and metabolic disturbance termed cachexia which accounted for death in 23% and 67% respectively, of the cases reported by Warren and Harnet. Indeed, Warren's findings further indicated that even in a number of cases where death had occurred due to definite anatomic causes, cachexia was nevertheless evident to either a greater or lesser degree. In contrast, Inagaki *et al* (1974) surveyed the causes of death in 816 cancer patients and reported that the majority of deaths occurred due to septicaemia, pneumonia, peritonitis, haemorrhage, infarction or organ failure, whilst only 10% of fatalities were due to severe emaciation.

1.2 Cachexia

The wasting phenomenon of cachexia is not unique to cancer patients, it is also seen as a manifestation of inflammation in infections such as Chagas' disease and AIDS. Major thermal injury and major or moderate surgical trauma may also result in the development of a catabolic state, as can diseases such as diabetes, chronic inflammatory bowel disease, chronic liver disease, chronic obstructive pulmonary disease, chronic cardiovascular disease and rheumatoid arthritis. However, in addition to being a major cause of death in

cancer patients, cachexia is also often the principal presenting symptom of the cancer itself with up to 80% (Albrecht and Canada, 1996) or even 100% (Wigmore *et al*, 1997a) of newly diagnosed patients having pre-existing weight loss. Indeed, there have even been recommendations in medical practice that any patient with unintentional weight loss should be evaluated for the presence of a malignancy (Marton *et al*, 1981).

The consequences of cachexia upon the cancer patient can be profound, with cachectic patients demonstrating significantly reduced survival and diminished responsiveness to chemotherapy when compared to non-cachectic patients (DeWys, 1986). Cachectic patients may also experience a propensity to infection and immobility (Wigmore *et al*, 1996a) and thus an impaired quality of life.

1.2.1 Incidence of Cachexia

The incidence of cachexia differs considerably depending largely upon tumour type. The lowest frequency of weight loss is observed in patients with favourable subtypes of non-Hodgkin's lymphomas, breast cancer, acute non-lymphocytic leukaemia and sarcomas (31-40%) whilst unfavourable non-Hodgkin's lymphoma, colon cancer, prostatic cancer and lung cancer demonstrate a 48-61% incidence of cachexia. The highest frequency of weight loss, however, is apparent in patients with pancreatic or gastric cancer (83-87%) (DeWys, 1986), and is often accompanied by the greatest degree of weight loss (>10%) (DeWys, 1985). Likewise, tumour extent may also affect the occurrence of cachexia, since breast cancer patients with metastatic disease of the liver, lung and bone demonstrated a 49% incidence of weight loss compared to a 31% incidence in patients with non-metastatic disease (DeWys, 1986).

1.2.2 Pathophysiology of Cancer Cachexia

Cachexia is characterised not only by a profound loss of body weight, but also by progressive weakness, early satiety, anaemia, oedema and disturbances of intermediary metabolism (Hall, 1979; Lawson *et al*, 1982). Typically, there is an overall decline in the

nutritional status of the patient, with factors such as anorexia, malabsorption and alterations in energy expenditure all contributing to this decline (Rich, 1987). Conventionally, weight loss or gain occurs as a consequence of an imbalance between energy expenditure and energy intake (Fearon and Carter, 1988). Though whether it is an increase in energy expenditure, a reduction in energy intake, or a combination of the two which is responsible for the development of the cachectic state has not been fully determined.

What does seem unlikely, however, is the theory that the tumour acts in a parasitic fashion, inducing host body weight loss by active competition for ingested nutrients. The rapidly growing, nutrient trapping foetus can represent the equivalent of >5% of the maternal body weight at birth, and yet will not engender a state of cachexia to occur in the mother. Conversely, a tumour burden of less than 0.01% may be sufficient to precipitate an overt loss of body weight (Williams and Matthaei, 1981) and even at autopsy few tumours in humans ever exceed 5% of host body weight (Heber and Tchekmedyan, 1992).

1.2.2.1 Anorexia and Cachexia

Any reduction in energy intake occurring in cachexia may arise due to a decrease in food consumption or assimilation. The development of anorexia is a common symptom of cancer, occurring either as an early manifestation of the disease or as the tumour proliferates and metastasises, with a large proportion of cancer patients exhibiting specific tumour-induced anorexia and inadequate dietary intake (Brennan, 1977). In the clinic, anorexia has been implicated as a contributory factor in the development of cachexia in lung (Staal-van den Brekel *et al*, 1994), pancreatic (Wigmore *et al*, 1997b), gastric and colon (Warnold *et al*, 1978) cancer patients.

The actual contribution of anorexia to the progression of cachexia is more defined if anorexia is present prior to diagnosis. After diagnosis psychological, emotional and therapeutic factors may all be implicated in the advancement of anorexia, and the actual role of the tumour becomes less distinct. The anorexia of cachexia is often associated with

changes in taste and smell sensation or learned food aversions (Giacosa *et al.*, 1996). Mechanical obstructions, especially in head and neck cancers, and malabsorption, a major cause of malnutrition in pancreatic cancer (Balducci and Hardy, 1985), may also be implicated. Whilst disturbances in the normal regulatory control of appetite, or prolonged feelings of satiety may further aggravate the imbalance of nutritional status. During acute disease anorexia may act beneficially, however, during chronic disease its effects are merely deleterious and in a cachectic cancer patient the development of anorexia simply exacerbates the pre-existing condition (Balducci and Hardy, 1985).

DeWys (1978) reviewed several studies performed on alterations in taste sensation and revealed that collectively the studies supported the conclusions that the probability of developing taste abnormalities increases with increasing tumour burden. Furthermore, he recorded that the most frequently observed alterations in taste sensation were: an increased threshold for sweet; a decreased threshold for bitter; an abnormally high recognition threshold for salt; a slight increase in the threshold for sour and a reduction in the recognition threshold for urea, producing a distinct aversion to meat. Additional factors such as age, colds, alcoholism and smoking may also produce alterations in taste sensation (Lindsey, 1986). Considered collectively, such alterations in taste sensation may decrease the pleasure of eating and thus reduce the inclination of the patient to eat, further elevating any underlying anorexia.

Specific chemotherapeutic drugs, such as cisplatin, cyclophosphamide and doxorubicin hydrochloride may also adversely affect food intake and thus further contribute to the development of cachexia. Sensations of nausea, or vomiting, experienced after drug administration will in themselves reduce the willingness of the patient to eat, and may also produce a syndrome of learned food aversions, where the patient begins to associate not only the drug treatment, but also certain recently ingested food stuffs with any side effects of the therapy (Lindsey, 1986).

The abnormalities in taste sensation occurring in the cancer patient may also serve to further exacerbate the cachectic state by decreasing the production, or activity, of the digestive enzymes and hence the availability of nutrients for intestinal absorption. Atrophic changes in gastrointestinal tract mucosa, due to a decreased rate of production of mucosal cells, a circumstance which may itself occur due to protein malnutrition and starvation and so is rather a consequence of cachexia than a cause, may also perpetuate patient weight loss. The cachectic state may also result in wasting of the patients' stomach muscles, an occurrence which may reduce the rate of gastric emptying thus prolonging sensations of satiety and so further decreasing appetite and intake (DeWys, 1979). It seems likely, therefore, that whilst malabsorption and gastrointestinal disturbances may contribute to the loss of host body weight seen in cachexia, they are more probably a manifestation of the pre-existing syndrome of wasting than its' primary cause.

Genuine alterations in the mechanisms responsible for feeding behaviour may also contribute to the anorexia of cancer cachexia. It has been proposed that the central control of energy balance is regulated by the brain via the multitude of neural, humoral and metabolic signals of the body's energetic status which it receives (Levin and Routh, 1996). Classically, the ventromedial hypothalamic nuclei (VHN) are regarded as the satiety centres of the brain whilst the lateral hypothalamic areas (LHA) are considered to be the feeding centres (Schwartz *et al*, 1995) with higher regions exerting modifying influences upon their abilities to induce or inhibit appetite development (Theologides, 1974).

The glucostatic theory postulates that one of the signals which may be important as a short-term regulator of appetite is blood glucose level. Reduced brain glucose utilisation is a powerful stimulant for food intake, therefore, when blood glucose levels fall the resultant decrease in glucose utilisation rate by the central nervous system (CNS) acts as an afferent signal to stimulate LHA and inhibit VHN neurones, thus provoking re-feeding (Schwartz *et al*, 1995). Conversely, insulin will decrease both blood glucose levels and dietary intake, possibly by acting as a lipostatic signal to the brain (Levin and Routh, 1996).

However, there have been suggestions that intraduodenal glucose levels may have a more important role in the regulation of feeding behaviour than blood glucose levels. Lavin *et al* (1996) investigated the effects of an intraduodenal versus an intravenous glucose infusion upon hunger and satiety in normal subjects. Their findings that there is an increase in satiety and a decrease in hunger following intraduodenal infusion, which is not seen after intravenous glucose infusion, question the validity of the glucostatic theory and suggest that appetite maybe regulated via intestinal glucoreceptors or osmoreceptors.

A whole host of neurotransmitters have been implicated in the long-term regulation of feeding. The monoamine serotonin, which is synthesised from the aromatic amino acid L-tryptophan, has been found to decrease food intake via its actions upon the VHN, and thus act as an anorectic (Cangiano *et al*, 1996a). Indeed, the anti-obesity drug fenfluramine acts as an anorectic through its ability to release serotonin (Kruk and Pycock, 1991). Catecholamines such as adrenaline, noradrenaline and dopamine may also have effects upon appetite, with dopamine exerting a stimulatory effect upon food intake via the LHA (Meguid *et al*, 1996).

Other proposed modulators of feeding behaviour include acetylcholine, γ -aminobutyric acid, glutamate, cholecystokinin (CCK), corticotrophin releasing hormone (CRH) and neuropeptide Y (NPY). The pancreatic polypeptide NPY acts upon the paraventricular nuclei (PVN) region of the hypothalamus to stimulate food intake, thus increasing body weight. It's production and release is stimulated by a reduction in hypothalamic insulin delivery due to an absence of nutrient stimulation of pancreatic islet β cells. Conversely, CRH potently inhibits food intake, thus inducing sustained anorexia and progressive weight loss. Activation of the hypothalamic-pituitary-adrenal (HPA) axis by NPY results in adrenocorticotropin hormone (ACTH) release and thus release of adrenal glucocorticoids which act via a negative feedback system to suppress CRH biosynthesis. However, prolonged activation of PVN CRH biosynthesis may prevent the normal compensatory

hypothalamic response to depleted body fuel stores and so result in adrenal insufficiency, glucocorticoid deficiency and thus sustained catabolism (Schwartz *et al*, 1995).

The obese gene product leptin has been demonstrated to reduce body weight, percent body fat, food intake and serum concentrations of glucose and insulin when administered intraperitoneally to obese *ob/ob* mice (Pelleymounter *et al*, 1995). When injected into the lateral ventricle of the brain leptin significantly attenuated food intake and body weight gain in lean mice whilst eliminating food intake and inducing body weight loss in *ob/ob* obese mice, thus indicating that leptin acts via one or more brain areas (Campfield *et al*, 1995). Indeed, leptin may act via a decrease in hypothalamic NPY expression and release, indicating that NPY may serve as the transducing mechanism for leptin action (Caro *et al*, 1996).

In conclusion, appetite in normal subjects is dependent upon many central and peripheral regulatory mechanisms. Perturbation of any one of these systems by either direct or indirect actions of the tumour may precipitate the development of anorexia, especially in cancer patients who are already experiencing therapeutic side effects, alterations in taste sensations or GI tract disturbances.

1.2.2.2 Alterations in Energy Expenditure Occurring in Cachexia

Resting energy expenditure (REE) may be defined as the minimum rate of energy expenditure following an overnight fast in an awake, relaxed, prone person, and represents a major determinant of total energy expenditure (Staal-van den Brekel *et al*, 1994). The role of an increase in energy expenditure in the development of the cachectic state has been the subject of much research. Any increase in basal metabolism which is not accompanied by a concomitant increase in caloric intake will generate a negative energy balance and thus necessitate utilisation of host tissues in order to satisfy metabolic demands. The normal adaptive mechanisms observed in simple starvation result in a decreased rate of energy expenditure in order to conserve body mass (Brennan, 1977). Furthermore, Kinney

(1995) reported that a 10% increase or decrease in body weight was accompanied by a concurrent rise or fall in energy expenditure, with REE falling significantly during periods of weight loss in both lean and obese individuals. Therefore, given the reduced body mass of the cachectic cancer patient, allied with an unaltered or reduced dietary intake, any increase in REE represents a failure of the normal survival mechanisms which occur during simple starvation.

Warnold *et al* (1978) studied energy balance and body composition in cancer patients in order to investigate the interrelationship between energy intake and energy expenditure in the development of cachexia. He reported that there were no significant differences between the daily energy intake of cancer patients and controls, whilst both daily energy expenditure and resting metabolic rate were significantly increased. Warnold concluded, therefore, that cachexia was a consequence of an increase in metabolic rate and energy expenditure allied with a failure of the normal adaptive mechanisms observed in simple starvation. Other workers, however, have found that cachexia occurs as a consequence of both increased energy expenditure and decreased caloric intake (Staal-van den Brekel *et al*, 1994; Wigmore *et al*, 1997b). Whilst Knox *et al* (1983) disputed that there is a uniform elevation of energy expenditure in the cancer patient, finding that of 200 cancer patients only 26% were hypermetabolic whilst 41% exhibited a normal metabolic rate and 33% were hypometabolic.

The mechanisms responsible for alterations in energy expenditure in cachexia have also been widely investigated. Smoking, tumour type, tumour stage and lung function have all been suggested to influence REE in lung cancer patients (Staal-van den Brekel *et al*, 1994). The heightened rates of glycolysis and oxidative metabolism which have been observed in cachectic cancer patients have also been implicated in the elevation of REE (Waterhouse, 1974). Lundholm *et al* (1982) also postulated that increased glucose turnover may produce an increase in metabolic rate in cancer patients. Whilst Hyltander *et al* (1991) reported an increased heart rate in hypermetabolic, weight losing cancer patients and hence concluded

that the increase in REE observed in cachexia is of true metabolic origin. Hyltander further correlated hypermetabolism to alterations in cardiac β -adrenoceptor expression and thus surmised that adrenergic factors may be responsible for the development of cachexia. Further studies revealed that of three experimental therapy regimes: indomethacin, a prostaglandin synthesis inhibitor; morphine, a powerful narcotic analgesic and propranolol, a β -adrenoceptor blocker, only propranolol significantly reduced REE, producing a decrease of 10% in cancer patients and 5% in normal controls (Hyltander *et al*, 1993). Thus lending support to the theory that an increase in the excretion of adrenaline and glucocorticoids may be the primary mechanism responsible for the observed elevation of energy expenditure, with cytokines such as interleukin-1 (IL-1), interleukin-6 (IL-6) and tumour necrosis factor (TNF- α) being less relevant.

Conversely, Staal-van den Brekel *et al* (1995) in a study of 87 lung cancer patients (77% of whom were hypermetabolic) recorded a significant correlation between increased REE and increased plasma concentrations of inflammatory mediators. These included soluble TNF- α receptors, soluble E-selectin, lipopolysaccharide binding protein and C reactive protein (CRP), with IL-6 concentrations also being significantly elevated in a group of 26 patients who had lost more than 10% of their original pre-illness stable weight (PSW), providing clinical evidence for the involvement of inflammation in the metabolic disturbances observed in cachectic lung cancer patients.

Likewise, Falconer *et al* (1994a) reported a significantly higher REE in cachectic pancreatic cancer patients compared to healthy controls, and observed that the most hypermetabolic patients were those demonstrating an ongoing hepatic acute phase protein response (APPR) which is characterised by an elevation in serum levels of CRP (≥ 10 mg/L). Further evidence for the potential role of cytokines in the elevation of REE was provided by Wigmore *et al* (1995) who demonstrated that administration of the non-steroidal, anti-inflammatory drug ibuprofen, which is known to inhibit some of the end organ effects of the pro-inflammatory cytokines, significantly reduced both REE and hepatocyte acute

phase protein expression. Wigmore *et al* (1997b) further observed that those patients demonstrating an APPR exhibited an accelerated rate of weight loss when compared to patients without an APPR. There was, however, a significant reduction in food intake (to 71% of normal) in patients with ongoing APPR, indicating that whilst anorexia is a contributory factor to the progression of cachexia, the APPR is probably a significant factor in the development of anorexia.

An elevation of energy expenditure, both with and without significant alterations in food intake, has been observed in animal models of cancer cachexia. A study into the mechanisms responsible for the induction of cachexia in the rat by T-cell leukaemia revealed increases in resting oxygen consumption, body temperature and brown adipose tissue (BAT) thermogenesis, indicative of hypermetabolism (Roe *et al*, 1996). These increases in energy metabolism and body temperature observed in this experimental model are believed to be a consequence of peripheral prostaglandin production (Roe *et al*, 1997). Mice bearing the cachexia-inducing MAC16 colon adenocarcinoma tumour model also demonstrated an increased REE (Plumb *et al*, 1991). Unlike other tumour models which only produce host weight loss when tumour burden reaches 3-15% of total host body weight (Bassukas and Maurer-Schultze, 1991), this murine cachexia model is capable of inducing a profound reduction in host body weight with a small tumour burden (Bibby *et al*, 1987) and so mimics the situation observed in human cancer patients. In the MAC16 model, energy expenditure per unit body weight increased to 123% of normal by the fourth week following tumour transplantation. This was accompanied by a gradual increase in both oxygen consumption and carbon dioxide production throughout the period of tumour growth (Plumb *et al*, 1991). The progression of cachexia in MAC16-bearing mice is believed to occur due to the production of lipolytic and proteolytic factors by the tumour (Beck and Tisdale, 1987). The actions of such catabolic factors may be responsible for the elevation of REE observed in this model.

1.2.2.3 *Metabolic Alterations in Cachexia*

1.2.2.3.1 *Carbohydrate Metabolism*

Profound disturbances in the normal metabolism of carbohydrate may be seen to occur in both cachectic and non-cachectic cancer patients. As early as 1930 Warburg had recognised that the majority of tumours exhibit a high rate of anaerobic glycolysis, which is not reduced in the presence of relatively high levels of oxygen. Whilst Lundholm *et al* (1982) observed that both the pool size and turnover rate of glucose are elevated in cancer patients when compared to controls, and Waterhouse and Kemperman (1971) noted a reduction in the rate of disappearance of glucose from the blood of cancer patients following glucose loading.

Normal glucose metabolism consists of three distinct biochemical pathways: glycolysis; the Citric acid, or Krebs, cycle and oxidative phosphorylation. During glycolysis glucose is converted via a series of intermediate metabolites to pyruvate which then undergoes oxidative decarboxylation to form acetyl coenzyme A (acetyl CoA). Acetyl CoA is then oxidised to carbon dioxide by the Citric acid cycle which it enters after condensing with oxaloacetate to produce citrate. Finally, energy, in the form of ATP, is liberated from the NADH and FADH₂ molecules formed during glycolysis and the Citric acid cycle by a process called oxidative phosphorylation, which involves the transfer of electrons from NADH and FADH₂ to oxygen via a series of electron carriers. A net energy gain of 36 molecules of ATP is produced from the complete oxidation of one molecule of glucose (Stryer, 1988).

The normal adaptations of the non tumour-bearing subject to conditions of semi-starvation involve a reduction in the ATP and oxygen requirements due to a reduction in oxidative metabolism (Waterhouse, 1974) and a conversion to a fat fuel economy (Brennan, 1977). Such conservational mechanisms, however, do not occur in the tumour-bearing host and so

both oxygen consumption and carbon dioxide production are inappropriately high (Waterhouse, 1974).

One of the alterations in carbohydrate metabolism commonly observed in cancer patients is glucose intolerance. In a study into carbohydrate metabolism in non-cachectic cancer patients Waterhouse and Kemperman (1971) observed that there were no differences in blood glucose levels between cancer patients and controls following an overnight fast. However, they noted that the rate of removal of glucose from the blood following the administration of a large glucose load was greatly reduced in cancer patients when compared to controls. Such glucose intolerance after oral or intravenous glucose administration was also recorded in both cachectic and non-cachectic cancer patients by Holroyde and Reichard (1981) who postulated that either insulin resistance or reduced pancreatic islet β cell insulin secretion may be the causative factor. Whilst Rofe *et al* (1994) reported that there was no correlation between tumour type or extent of host wasting and glucose intolerance, they speculated that as insulin resistance is also a clinical characteristic of simple starvation, then the insulin resistance observed in cachectic cancer patients may be simply a feature of patient starvation rather than a manifestation of the tumour itself.

The rate of slow oxidative metabolism of glucose has also been observed to be impaired in cancer patients with significant amounts of ingested glucose being disposed of by means other than oxidation (Waterhouse, 1974; Rofe *et al*, 1994). Such an impairment may be due to the high rates of anaerobic glycolysis and Cori cycle activity which have been widely observed in cancer patients (Waterhouse and Kemperman, 1971; Waterhouse, 1974; Buzby *et al*, 1980; Holroyde and Reichard, 1981; Lundholm *et al*, 1982). Under aerobic conditions pyruvate produced by glycolysis is shunted directly into the Citric acid cycle. However, in anaerobic conditions, such as those found in actively contracting skeletal muscle or hypoxic tumour tissue, the rate of production of NADH and pyruvate from glycolysis will far exceed their rate of oxidation by the Citric acid cycle. In such

circumstances of oxygen deprivation the Cori cycle will operate, the sole purpose of this cycle being to regenerate NAD^+ and glucose, thus permitting the continuance of glycolysis.

The Cori cycle consists of the conversion of pyruvate and NADH from glycolysis to lactate and NAD^+ by the enzyme lactic dehydrogenase, and the subsequent reconversion of lactate back to pyruvate and hence, via gluconeogenesis in the liver, to glucose (Stryer, 1988). Lactate itself represents a metabolic 'dead end' and must be reconverted to pyruvate before further metabolism can occur. Additionally, the Cori cycle is energy consuming (Lundholm *et al*, 1981) requiring 6 mol ATP for gluconeogenesis, whilst producing only 2 mol ATP from glycolysis, and so further exacerbates the energy imbalance observed in cachectic cancer patients by contributing to the elevation of REE (Waterhouse, 1974; Holroyde and Reichard, 1981; Lundholm *et al*, 1982). Indeed it has even been suggested that the inhibition of gluconeogenesis may prove to be an effective strategy for preventing the progression of cachexia (Gold, 1968).

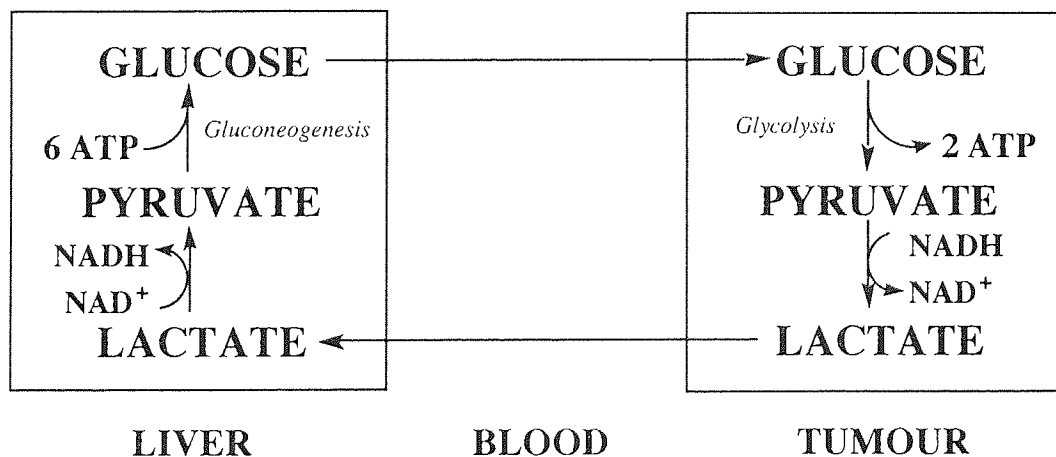


Figure 1.1:- The Cori Cycle

Whilst results obtained in metabolic studies of tumour-bearing animals are not necessarily representative of the metabolic reactions occurring in cancer patients, alterations in carbohydrate metabolism have also been reported in mice bearing experimental tumours,

and in tumour cells grown *in vitro*. The TLX-5 lymphoma produced significant alterations in free CoA, acetyl CoA and citrate 24h after tumour inoculation, whilst similar changes were reported in mice bearing the C₃H mammary carcinoma once the tumour became palpable. A reduction in the liver content of free CoA and α -oxoglutarate was also observed in mice bearing Sarcoma 180. Such alterations probably elevate gluconeogenesis in the tumour-bearing host and thus affect REE (McAllister *et al*, 1982). NMRI mice transplanted with tumours induced by exposure to 1,2-dimethylhydrazine hydrochloride (MAC16 tumour-bearing mice) demonstrated profound hypoglycaemia (McDevitt and Tisdale 1992) and a high ratio of anaerobic to aerobic glucose utilisation *in vitro* (Tisdale and Brennan, 1986). Further studies revealed this tumour to be the second major consumer of glucose *in vivo* after the brain (Mulligan and Tisdale, 1991a) thus illustrating the high metabolic demand which a tumour may impose upon its host.

In a study into the impairment of respiration by glycolysis in the Lewis lung carcinoma, Miralpeix *et al* (1990) reported low rates of basal respiration with low rates of oxygen uptake and cytochrome-c oxidase activity, and high rates of lactate production in tumour cells cultured *in vitro*. These alterations have been associated with a reduction in the number of mitochondria present in tumour cells, a change which impairs the respiratory capacity of the cell thus increasing the occurrence of anaerobic glycolysis, and hence lactate production, in tumour cells (Miralpeix *et al*, 1990). A high rate of anaerobic glycolysis was also observed in Ehrlich ascites tumour cells *in vitro* (Spector and Steinberg, 1966), and in the rapidly growing rat hepatoma cell line AS-30D, indicating that this is not an uncommon phenomenon.

It may be concluded, therefore, that significant alterations in the normal pathways for carbohydrate metabolism commonly occur in cancer patients, experimental tumour models and tumour cells maintained *in vitro*. Insulin insensitivity, preventing the storage of exogenous glucose, coupled with enhanced anaerobic glycolysis via the futile Cori cycle,

results in a reduction in the efficiency of glucose utilisation by the tumour-bearing host, and may contribute to an elevation of REE, thus further exacerbating any underlying cachexia.

1.2.2.3.2 Protein Metabolism

Whilst loss of body fat may represent the greatest contribution to the reduction in host body weight observed in cachexia, the loss of body protein is of far greater prognostic significance. A profound depletion of body cell mass, in proportion to total weight loss has been observed in cachectic cancer patients (Moley *et al*, 1987; Falconer *et al*, 1994a), whilst body composition analyses have revealed a significant reduction in lean body mass in animals bearing experimental tumour models (Clark and Goodlad, 1971; Svaninger *et al*, 1983; Pain *et al*, 1984; Bibby *et al*, 1987; Tessitore *et al*, 1987). Unlike carbohydrates and fatty acids excess dietary amino acids cannot be stored by mammals, nor are they excreted, instead they must be utilised as metabolic fuels.

Deamination of amino acids in the liver results in the formation of an amino group, which is subsequently excreted in the form of urea, and a carbon skeleton which is further converted into either acetyl CoA, acetoacetyl CoA, pyruvate, or an intermediate of the Citric acid cycle. Hence excess amino acids will be degraded in the liver to form major metabolic intermediates which may then be utilised in gluconeogenesis, or oxidised via the Citric acid cycle to yield energy (Stryer, 1988).

During uncomplicated starvation there is a rapid early proteolysis with mobilisation of amino acids from muscle at a rate equivalent to a loss of approximately 75g of muscle protein per day. The continuation of such a rate of muscle catabolism under conditions of prolonged starvation would result in the loss of 30% of total muscle mass in 20 days. Adaptations of the normal subject to conditions of starvation will minimise muscle wasting by converting to a fat fuel economy, ensuring that only 2% of total body muscle is lost in 20 days (Brennan, 1977). Such muscle conserving mechanisms, however, are not manifest in cachectic cancer patients who demonstrate continued muscle wasting, allied

with an increased conversion of radiolabelled alanine to glucose by gluconeogenesis (Waterhouse *et al*, 1979). Indeed, a 72% stimulation of gluconeogenesis from amino acids has been reported in tumour-influenced hepatocytes exposed to exogenous alanine (Roh *et al*, 1984).

Hypoalbuminaemia, the degree of which correlated with the extent of the cancer, and also with the occurrence of malnutrition, has frequently been observed in tumour-bearing patients and animals (Costa, 1977). However, the alterations in protein metabolism noted in cachectic individuals cannot be accounted for by malnutrition alone. In an investigation into the role of malnutrition in the development of cachexia, Tessitore *et al* (1993b) reported a decrease in the protein content of the gastrocnemius muscle and the heart by 10 days post tumour implantation, and postulated that whilst pair-fed controls also lost body weight this could probably be attributed to a reduction in protein synthesis, rather than an increase in protein degradation such as that observed in cachectic animals. These findings were further endorsed by Li and Goldberg (1976) who reported a decrease in muscle RNA content and hence decreased protein synthesis in the muscles of fasted rats.

Muscle wasting may occur as a consequence of increased muscle catabolism, decreased muscle protein synthesis, or a combination of the two. Many studies have employed experimental models of cancer cachexia in an attempt to determine the mechanism of muscle loss in host wasting, and their findings have been disparate. Clark and Goodlad (1971) compared the effects of the Walker 256 carcinoma upon two different muscle types, the 'white' gastrocnemius muscle and the 'red' soleus muscle. Whilst both muscles decreased in weight and protein content during tumour progression, with such losses occurring almost entirely from the myofibrillar protein fraction, the gastrocnemius demonstrated a greater degree of wastage than the soleus muscle. Studies employing radiolabelled amino acids further revealed that the incorporation of amino acids into new muscle protein was attenuated in the gastrocnemius muscle during tumour growth, but unaffected in the soleus muscle (Clark and Goodlad, 1971). Hence, whilst decreased rates of muscle protein

synthesis may account for the wastage of white muscle fibres, the depletion of red skeletal muscle mass is rather the consequence of an increased rate of muscle catabolism. The debate over the relative contributions of elevated catabolism and depressed synthesis to muscle wasting continues, with some studies implicating increased rates of muscle protein degradation with no alteration in protein synthesis (Tessitore *et al*, 1987; Llovera *et al*, 1995), whilst other workers have reported decreased synthesis alone to be the primary cause of muscle wasting (Rennie *et al*, 1983; Svaninger *et al*, 1983; Lopes *et al*, 1989), and both decreased synthesis and enhanced proteolysis have been implicated in other experimental models of cancer cachexia (Smith and Tisdale, 1993a). Furthermore, whilst host muscles atrophy during cachexia the liver has often been observed to increase in size (de Blaauw *et al*, 1997) with elevated synthesis of certain hepatic acute phase proteins occurring, even in the presence of profound muscle catabolism (Falconer *et al*, 1994a; de Blaauw *et al*, 1997), indicating that the tumour may have differing effects upon different protein synthesis pathways.

Investigations into the exact mechanisms of elevated muscle proteolysis have suggested the involvement of the extralysosomal ATP- and ubiquitin-dependent proteases in the wasting of muscle mass in cancer cachexia (Baracos *et al*, 1995; Llovera *et al*, 1995). This energy-dependent pathway is characterised by an increased expression of polyubiquitin mRNA in tumour-bearing animals, which is not manifest in pair-fed controls, accompanied by increased levels of ubiquitin conjugated proteins (Baracos *et al*, 1995). This pathway appears to be the mechanism of muscle protein breakdown in various catabolic states including fasting and metabolic acidosis, as well as cancer cachexia (Mitch and Goldberg, 1996).

The presence of factors with proteolytic activity has also been reported in both the urine and plasma of cachectic cancer patients (Belizario *et al*, 1991; Todorov *et al*, 1996a), the plasma of patients with sepsis or trauma (Clowes *et al*, 1983), and in an extract of the cachexia inducing MAC16 tumour (Beck and Tisdale, 1987). The purification, and subsequent

intravenous administration, of a MAC16-derived proteolytic factor to non tumour-bearing female NMRI mice revealed an elevation in muscle wasting due to enhanced proteolysis involving activation of the ATP-ubiquitin-dependent pathway (MJ Lorite, personal communication). Northern blot analysis of muscles from both MAC16 bearing and proteolysis-inducing factor (PIF) treated mice revealed a similar pattern of increased ubiquitin mRNA expression and elevated levels of ubiquitin conjugated proteins to that reported by Baracos *et al* (1995).

It seems probable, therefore, that both depression of protein synthesis and elevation of protein catabolism are responsible for the loss of host lean muscle mass in cancer cachexia, with mobilised amino acids being utilised either as an additional fuel source, or to provide structural materials for tumour growth. Enhanced rates of muscle catabolism are presumably a consequence of the production of PIF by the tumour and PIF may also act to suppress protein synthesis, or to redirect host protein synthesis away from muscle proteins towards certain hepatic acute phase proteins. Loss of muscle will have significant effects upon the host, especially with regard to quality of life, since muscle wasting will be largely accountable for the feelings of lethargy experienced by the cachectic cancer patient, whilst wasting of GI tract musculature may also exacerbate any anorexia experienced by the patient thus worsening nutritional status still further.

1.2.2.3.3 Lipid Metabolism

Fat represents the major energy store of the body with complete oxidation of fatty acids yielding three times more ATP than the oxidation of the equivalent amount of glucose (Stryer, 1988). A host of techniques have been developed to enable the study of *in vivo* adipose tissue metabolism, including the use of isotopic tracers, microdialysis, measurements of circulating levels of glycerol and free fatty acids (FFA) and anthropomorphic measurements such as triceps skinfold thickness, whilst lipolysis, glucose transport and triacylglycerol (TAG) turnover may be measured *in vitro* using either tissue fragments or isolated whole adipocyte preparations (Arner, 1995).

Energy is liberated from fatty acids by a process known as β -oxidation which occurs in the mitochondria of cells. Prior to entry to the mitochondria fatty acids are activated via the formation of a thioester linkage with CoA to form acyl CoA which subsequently undergoes a series of oxidation and hydration reactions involving the sequential removal of two carbon units from the carboxyl end of the fatty acid in the form of acetyl CoA. Providing that the metabolism of fat and carbohydrate is balanced, acetyl CoA will be further oxidised via the Citric acid cycle. However, during periods of starvation, when oxidation of fatty acids predominates, oxaloacetate, which is required for the entry of acetyl CoA to the Citric acid cycle, will be utilised for gluconeogenesis, thus precluding complete oxidation of mobilised fat. Under such circumstances, acetyl CoA will be transported to the liver and converted into acetoacetate and D-3-hydroxybutyrate which are collectively known as ketone bodies. Ketone bodies represent a proportion of the normal metabolic fuels of respiration and energy provision being preferentially metabolised by the heart and renal cortex even when glucose is plentiful. Conversely, the brain favours glucose as its main metabolic fuel, however, it too can adapt to utilise ketone bodies during periods of prolonged starvation (Stryer, 1988), and there have been suggestions that tumours may also utilise ketone bodies as a source of metabolic fuel (Sauer and Dauchy, 1983). This is especially true of hepatic carcinomas which demonstrated the ability to exploit both D-3-hydroxybutyrate (Ohe *et al*, 1967) and acetoacetate (Fenselau *et al*, 1975) as metabolic fuels. Conversely, tumours of non-hepatic origin possess a limited capacity for acetoacetate metabolism due to low succinyl-coenzyme A : acetoacetate CoA transferase levels (Tisdale and Brennan, 1983) and so will preferentially utilise glucose, even under conditions of chronic starvation. Indeed, the inability of cultured, transformed lymphoblasts to utilise D-3-hydroxybutyrate has been implicated in the inhibition of cellular proliferation (Magee *et al*, 1979).

The principal perturbations in lipid metabolism observed in cachectic cancer patients are an elevation in total fatty acid turnover due to increased lipolysis coupled with increased whole body fatty acid oxidation, resulting in severe depletion of host adipose tissue stores. This depletion of host lipid stores may occur due to either a reduced rate of fatty acid deposition,

or an increased rate of mobilisation (Thompson *et al*, 1981). In addition studies on whole body lipolysis and triglyceride-fatty acid (TG-FA) cycling in cachectic oesophageal cancer patients revealed an increase in this futile cycle (Klein and Wolfe, 1990). Elevated plasma levels and turnover rates of both FFA and glycerol have been reported in hypermetabolic cancer patients with an average weight loss of 13% (Legaspi *et al*, 1987). There was no evidence of extensive re-esterification of mobilised FFA in these patients with 53% of mobilised lipids undergoing oxidation and 29% of the total FFA turnover being converted to carbon dioxide, indicating the utilisation of fatty acids as an energy source by the patients (Legaspi *et al*, 1987).

Alterations in lipid metabolism in cachectic cancer patients have often been related to the presence of anorexia (Costa, 1977), and the theory advanced that as a similar mode of lipid depletion was observed both in cachectic cancer patients and cachectic non-cancer patients, such alterations may simply be the consequence of the patients' nutritional status (Klein and Wolfe, 1990). However, alterations in lipid metabolism have also been reported without a significant reduction in food intake (Costa, 1977), and hence it has been postulated that factors capable of mediating of lipid metabolism may be produced either by the tumour itself, or by the host in response to the presence of a malignancy (Masuno *et al*, 1981). The involvement of lipid-mobilising factors (LMF) in host adipose tissue depletion has been demonstrated in cachectic cancer patients with a variety of malignancies (Theologides, 1976; Kitada *et al*, 1981; Groundwater *et al*, 1990; McDevitt *et al*, 1995; Gercel-Taylor *et al*, 1996). However, other workers have proposed that the stress to the patient produced by the presence of the tumour may result in an elevation of circulating levels of catecholamines which, allied with host insulin resistance, could increase lipolysis and hence deplete lipid stores (Klein and Wolfe, 1990). Conversely, Jeevanandam *et al* (1986) postulated that body fat depletion in cachectic cancer patients is rather the consequence of defective dietary fat absorption, or decreased *de novo* lipogenesis rather than the outcome of an elevation in adipose tissue lipolysis. Furthermore, both elevation of lipolysis and

depression of lipogenesis may be implicated as mechanisms of adipose tissue wasting in patients exhibiting insulin resistance (Lundholm *et al*, 1981).

Animal studies have revealed that several cachexia-inducing tumour models are capable of inducing profound alterations in lipid metabolism in recipient animals, with alterations in both deposition and mobilisation of host fat stores being implicated as the causative factors. Plasma levels of TAG and FFA have been observed to decrease in some tumour models such as the MAC16 colon adenocarcinoma (Bridson *et al*, 1991) whilst hypertriglyceridaemia was noted in mice bearing the Ehrlich ascites carcinoma (Balint, 1991). Kannan *et al* (1980) proposed that a reduction in lipogenesis in the host tissues of mice bearing the Ehrlich ascites carcinoma may be the primary mechanism underlying the depletion of body fat observed in cachexia. A decrease in the normal rate of post prandial lipogenesis was also observed in the liver and adipose tissue of male rats bearing a cachexia-inducing Leydig cell tumour (Emery *et al*, 1993). This reduction in lipogenesis correlated with the depression of adipose tissue lipoprotein lipase (LPL) activity observed in this cachexia model (Obeid and Emery, 1993) and in mice bearing the ESR-586 tumour model (Thompson *et al*, 1981). Conversely, substantial LPL activity could be demonstrated within the tumour itself (Thompson *et al*, 1981; Obeid and Emery, 1993) indicating a diversion of fatty acids away from storage by the host towards utilisation by the tumour.

The Krebs-2 carcinoma induced a triphasic loss of body fat when transplanted into adult male Swiss mice which consisted of an early acute phase of fat depletion occurring within one week of tumour implantation followed by a period of continuous tumour growth without further significant fat loss, and finally a renewal of lipid-mobilisation during the pre-mortal period (Costa and Holland, 1962). Whilst anorexia may play a role in host adipose tissue depletion in this tumour model, the pattern of early weight loss implies that a different mechanism was responsible. This was further supported by the findings of Lundholm *et al* (1980) who reported that whilst tumour-bearing cachectic mice were also

anorexic, their loss of body fat was nevertheless far more extensive than that observed in pair fed animals, indicating the involvement of factors other than simple starvation.

Tumour-derived lipid-mobilising factors have been implicated in the pathogenesis of several cachexia inducing tumour models (Kralovic *et al*, 1977; Kitada *et al*, 1980; Masuno *et al*, 1981; Beck and Tisdale, 1987; Stovroff *et al*, 1989; Balint, 1991) whilst increased levels of basal lipolysis have been reported in the adipose tissue of both rats bearing the Walker 256 carcinoma (Kralovic *et al*, 1977) and mice bearing the preputial gland tumour ESR-586 (Thompson *et al*, 1981), implying that an increased rate of FFA mobilisation may be the principal mechanism of host lipid depletion in these experimental tumour models.

Investigations into the mechanisms responsible for the inducement of cachexia by the MAC16 colon adenocarcinoma have revealed a reduction in plasma TAG levels, irrespective of tumour burden, accompanied by an overall decrease in plasma FFA concentrations and adipose tissue LPL activity (Bridson *et al*, 1991). Evidence that this tumour elevated the lipid requirements of the cachectic host was provided by observations that brain glucose utilisation was depressed whilst there was an increased metabolism of D-3-hydroxybutyrate (Mulligan *et al*, 1992a). Extensive host adipose tissue depletion was also detected in MAC16 tumour-bearing mice *post mortem* (Bibby *et al*, 1987) and has been correlated to the production by the tumour of a specific factor with lipid-mobilising activity (Beck and Tisdale, 1991). Raised hepatic fatty acid synthase activity has also been recorded (Mulligan and Tisdale, 1991a) indicating increased *de novo* lipogenesis. Viewed collectively, the observations of the depletion of host lipid stores allied with elevated utilisation of ketone bodies by the brain, increased *de novo* lipogenesis and decreased plasma FFA levels would appear to indicate that the metabolic demands of the MAC16 bearing host and tumour are not sufficiently provided for by dietary intake alone, despite the absence of anorexia in this tumour model. Therefore, the tumour has finely developed mechanisms which enable it to obtain lipids from host tissues in order to fulfil its own metabolic and structural requirements.

The alterations in lipid metabolism observed in the tumour-bearing host would appear to indicate an increased demand for fatty acids by the growing tumour, allied with a requirement by the host for alternative fuel supplies due to the high glucose requirements of the tumour. The production of factors by the tumour to induce the mobilisation of host adipose tissue stores, thus depleting body fat, probably serves to facilitate tumour growth and metabolism via an increased availability of such materials.

It would seem probable, therefore, that the disturbances in carbohydrate, protein and lipid metabolism observed both in cachectic cancer patients and in animals bearing experimental tumour models occur as a consequence of the metabolic and structural demands of the growing tumour allied with the failure of the host to adapt appropriately to conditions of semi-starvation. The poor vasculature, and hence hypoxic nature of many solid tumours necessitates anaerobic metabolism thus obliging the host to operate the energy demanding Cori cycle in order to recycle tumour produced lactate and regenerate NAD^+ , enabling continued glycolysis. The breakdown of lean body mass, most probably by tumour produced proteolytic factors serves to provide amino acids for the formation of structural proteins to enable tumour growth, and metabolic intermediates for continued gluconeogenesis and ketogenesis. Such depletion of host muscle mass will severely debilitate the cancer patient producing sensations of weakness and lethargy. Lipid mobilisation, which like protein breakdown is largely tumour-controlled, will also provide materials to capacitate tumour growth, indeed some fatty acids may even serve to promote tumour development. Mobilised lipids may also be required by the host as an alternative energy source as the metabolic demands of the tumour increase, necessitating host metabolism of lipid-derived ketone bodies which may even provide a supply of energy to certain tumours. In conclusion, the tumour, whilst not directly competing with the host for ingested nutrients, will impose an increased metabolic demand upon the patient, which, if not provided for by an increased dietary intake, will result in the mobilisation of host tissues in order to meet its own metabolic and proliferative demands.

1.2.3 Humoral Mediation of Cancer Cachexia

The possible role of a circulating cachexia-inducing factor has been extensively investigated. In 1958 Chalmers *et al* postulated that the excretion of a factor with lipid-mobilising activity in the urine of cancer patients indicated a hormonal involvement in host body weight loss. Anorexigenic and cachexigenic tumour-derived peptides have subsequently been reported in both the urine and serum of cancer patients (Theologides, 1976; Kitada *et al*, 1981; Masuno *et al*, 1984; Groundwater *et al*, 1990; Knapp *et al*, 1991), experimental models of cachexia (Kralovic *et al*, 1977; Kitada *et al*, 1980; Thompson *et al*, 1981; Beck and Tisdale, 1987; Tessitore *et al*, 1993b) and in the media of cultured tumour cells (Kitada *et al*, 1981; Taylor *et al*, 1992). Confirmation of the humoral nature of cachexia was provided by Norton *et al* (1985) who demonstrated that the non tumour-bearing half of a parabiotically joined pair of rats exhibited identical symptoms of emaciation as its tumour-bearing partner, without any evidence of metastasis of the primary tumour. The actual factor responsible for this humoral effect has been the subject of much debate, and several candidates including cytokines and novel lipolytic and proteolytic factors have been proposed.

1.2.3.1 Tumour Necrosis Factor (TNF- α) / Cachectin

Tumour necrosis factor (TNF- α), or cachectin, is a 17kDa proinflammatory cytokine produced by cells of the reticuloendothelial system in response to endotoxin challenge, which has been demonstrated to initiate host wasting in disease states involving chronic inflammation. TNF- α has been implicated in: the induction of pyrogen fever; the synthesis of hepatic acute phase proteins; increased vascular permeability; fibroblast proliferation; the induction of IL-6 and IL-8 and the activation of both T-cells and B-cells.

The ability of TNF- α to induce necrosis in tumours of cancer patients who developed certain bacterial infections was first noted in the late 19th century by surgeon William Coley, since when TNF- α has been demonstrated to be responsible for: the wasting associated with acute infection with *Trypanosoma cruzi*, the causative agent of Chagas'

disease (Truyens *et al*, 1995); the elevated acute phase protein response seen in chronic inflammatory bowel disease (O'Connell *et al*, 1994) and the cachexia of both chronic heart failure (Zhao and Zeng, 1997) and adjuvant arthritis (Roubenoff *et al*, 1997). Beutler and Cerami (1986) reported the isolation of a factor which they termed cachectin from rabbits in the final stages of infection with *Trypanosoma brucei*, who demonstrated lipaemia and severe wasting. The principal finding associated with the development of cachexia in *T brucei* infected rabbits was an increase in serum lipid levels which occurred as a consequence of reduced host LPL activity. Subsequent sequence analysis of mouse cachectin, and cloning of the cDNA responsible for its expression, revealed that cachectin and TNF- α were actually identical molecules (Beutler and Cerami, 1988).

The mechanisms of action of TNF- α in altering host metabolism have been widely investigated. Bernstein (1996) suggested that TNF- α could be implicated in the neural mediation of food aversions, whilst an increased rate of proteolysis and a depressed rate of protein synthesis in skeletal muscle was observed upon both acute (Garcia-Martinez *et al*, 1993) and chronic (Llovera *et al*, 1993; Ling *et al*, 1997) administration of TNF- α to rats, allied with an increased liver protein anabolism (Ling *et al*, 1997). Alterations in lipid metabolism have also been reported upon TNF- α administration, with depressed LPL activity and increased lipolysis being reported in cultured fat cells (Grunfeld and Feingold, 1996; Hauner *et al*, 1995).

The involvement of TNF- α in the development of cachexia was further investigated by Oliff *et al* (1987) who produced a TNF- α secreting cell line which consisted of Chinese Hamster Ovary (CHO) cells containing a clone of the TNF- α gene. When transplanted intramuscularly into nude mice this tumour model induced profound cachexia (17.6% loss of total body weight), which was manifested as extensive muscle wasting, loss of host adipose tissue and anorexia. Thus cachexia induced by this tumour model appeared similar to the situation observed in cachectic cancer patients. The presence of TNF in the serum of tumour-bearing rats and cancer patients has been difficult to detect: Knapp *et al* (1991)

reported significantly increased serum concentrations of TNF- α in cachectic stage IV breast cancer patients. Conversely, Socher *et al* (1988) studied 19 cancer patients with 8-40% loss of initial body weight over the preceding 2-9 months, and failed to detect elevated serum levels of TNF- α , whilst Stovroff *et al* (1989), in a study of sarcoma-bearing rats were only able to detect an elevation of serum TNF- α levels as cachexia and tumour burden progressed. Such findings could, however, reflect a lack of sensitivity of the assay method employed and so may not conclusively eliminate TNF- α as the causative agent of cancer cachexia. Falconer *et al* (1994a), isolated polymorphonuclear cells from cachectic pancreatic cancer patients undergoing an elevated APPR and discovered an increased secretion of TNF- α , which could explain the elevated REE which was also observed in this patient group. It has also been postulated that very low, undetectable circulatory levels of TNF- α may be sufficient to induce cachexia in pre-sensitised patients (Garcia-Martinez *et al*, 1997). Furthermore, TNF- α mRNA has been discovered at the tumour sites of BALB/c, nude and SCID mice bearing clone 5 of the colon 26 tumour model (Yasumoto *et al*, 1995).

Administration of TNF- α to NMRI mice also resulted in a dose-dependent reduction in body weight which was directly proportional to the development of anorexia (Mahony and Tisdale, 1988). Mahony and Tisdale further reported that acute TNF- α administration induced hypoglycaemia, reduced circulatory FFA and elevated plasma TAG which was accompanied by a reduction in adipose tissue mass and body water content. Conversely, chronic TNF- α administration resulted in weight loss during the first 24h post injection, but thereafter body weight increased to that of pair-fed controls. A comparison of the weight loss induced by TNF- α with that produced by a cachexia-inducing tumour model, the MAC16 colon adenocarcinoma, indicated that whilst tumour-bearing mice did not demonstrate any anorexia the degree of weight loss due to TNF- α administration was directly proportional to the reduction in food consumption previously observed (Mahony *et al*, 1988), indicating that TNF- α had no role in the induction of cachexia by this tumour model. Studies into the metabolic effects of TNF- α revealed a reduced rate of carbon

dioxide production from radiolabelled glucose, but no alteration in the rate of fatty acid utilisation, accompanied by elevated plasma levels of ketone bodies and a reduction in rectal body temperature, indicative of adaptations to conditions of simple starvation (Mahony and Tisdale, 1990). Furthermore, administration of anti-TNF- α antibodies to mice bearing the MAC16 tumour had no effect upon either the development of cachexia, or tumour growth (Mulligan *et al*, 1992b). Thus TNF- α is not the mediator of cachexia induced by the MAC16 tumour model.

Therefore, whilst TNF- α may be implicated in the development of cachexia in certain disease states, and may even be involved in the pathogenesis of certain tumours, it seems very unlikely that TNF- α is a universal mediator of the development of cancer cachexia. Rather TNF- α is anorectic, inducing weight loss appropriate to the reduction in food intake produced by its administration. However, whilst the presence of elevated TNF- α alone may not be sufficient to engender the development of the cachectic state, it should be noted that TNF- α is capable of inducing the production of other cytokines by the tumour-bearing host and so may possibly play a regulatory role in the induction of cachexia.

1.2.3.2 Interleukin 1 (IL-1)

The possible role of IL-1 in the humoral mediation of cancer cachexia is indistinct. IL-1 has been implicated in the interactions occurring between host macrophages and colon 26 carcinoma cells, and so may be involved in either the initiation or exacerbation of the cachectic state (Strassman *et al*, 1993). Furthermore, administration of IL-1 to normal animals has been demonstrated to induce symptoms usually associated with the cachectic state, with alterations in post absorptive glucose metabolism, weight loss and skeletal muscle catabolism being recorded (Ling *et al*, 1997). Likewise, Argiles *et al* (1989) reported a 40-50% reduction in intestinal lipid absorption, a two to three fold decrease in lipid oxidation, a suppression of the uptake of lipids into adipose tissue, and hypertriglyceridaemia upon intravenous administration of IL-1 β .

However, since IL-1 is known to incite the production of IL-6 (Strassman *et al*, 1993) and the expression of IL-6 mRNA (Yasumoto *et al*, 1995), it is difficult to distinguish the effects of IL-1 from those of IL-6. Similarly, although IL-1 is known to promote the production of hepatic acute phase proteins, which have often been associated with the progression of cachexia, inhibit the actions of LPL, and to induce lipolysis *in vitro*, there is no conclusive evidence of an elevation in the serum levels of IL-1 in mice bearing a cachexia-inducing clone of the colon 26 carcinoma (Fujimoto-Ouchi *et al*, 1995) nor has the expression of IL-1 mRNA in colon 26 cells cultured *in vitro* been confirmed (Yasumoto *et al*, 1995). Moreover, IL-1 receptor antagonist (IL-1RA) and anti-IL-1 monoclonal antibody (MAb) were demonstrated to attenuate cachexia via a significant reduction in tumour IL-6 production (Strassman *et al*, 1993; Yasumoto *et al*, 1995). Conversely, Costelli *et al* (1995) reported that IL-1RA was ineffective in reversing the cachexia induced in rats by the ascites hepatoma Yoshida AH-130, and hence postulated, that in their model at least, IL-1 was of no importance in the development of cachexia.

It seems probable, therefore, that any role which IL-1 may play in the development of tumour-induced cachexia is likely to be the result of its modulatory effects upon IL-6 production, rather than a direct effect of IL-1 itself. However, since the transfection of IL-1RA cDNA into a cachexia inducing clone of the colon 26 carcinoma failed to abolish the capacity of the clone to produce IL-6 and induce cachexia (Yasumoto *et al*, 1995), it would appear that additional factors are also required for the development of the cachectic state.

1.2.3.3 Interleukin 6 (IL-6)

The pro-inflammatory cytokine IL-6 is known to play a pivotal role in the response of the host to tissue injury, with elevated circulating levels of IL-6 being recorded during infection, auto-immune diseases and neoplasia (Sehgal, 1990). IL-6 is known to be capable of inducing lipolysis, proteolysis and hepatic APPR, and so appears a promising candidate for the humoral mediation of cancer cachexia. The actual significance of IL-6 in

the development of cancer cachexia is, however, less clearly defined with many contradictory reports regarding the relationship existing between IL-6 and host weight loss.

Strassman *et al* (1992) derived a cachexigenic tumour model from the colon-26 adenocarcinoma, capable of inducing skeletal muscle wasting and loss of host body fat as an early effect with a small tumour burden (<3%), without the induction of anorexia, and reported increased plasma levels of IL-6 which correlated with the progression of cachexia. Resection of the primary tumour, or administration of anti-IL-6 MAb, resulted in a regression of the cachectic state accompanied by a concomitant decrease in plasma IL-6 concentration. Strassman *et al* thus concluded that IL-6 played a major role in the development of cachexia in mice bearing that particular clone of the colon-26 adenocarcinoma. Increased circulating levels of IL-6 were also postulated to be the causative mechanism of the cachexia observed in T-cell leukaemic rats (Roe *et al*, 1997).

Increased production of IL-6 in tumour-bearing mice has been observed to occur in a variety of host tissues. Elevated expression of IL-6 mRNA, which may be directly correlated to increased circulatory bioactive IL-6, has been demonstrated in the liver, kidneys, small intestine and spleen, with the tumour itself representing the major source of plasma IL-6 (Lonroth *et al*, 1994). High serum levels of IL-6 have also been correlated to poor prognosis in patients with primary epithelial ovarian cancer, with suggestions that IL-6 may act as a growth factor, promoting the growth and metastasis of the primary tumour (Scambia *et al*, 1995). Furthermore, whilst no significant reciprocity was found to exist between the development of cachexia and serum concentrations of IL-6 and CRP in patients with non-small-cell lung cancer, it was possible to correlate elevated serum CRP levels with raised serum IL-6 (Scott *et al*, 1996). This relationship between elevated serum IL-6 concentration and hepatic APPR was also noted in colorectal cancer patients (Fearon *et al*, 1991). Likewise, Falconer *et al* (1994a) demonstrated an increased secretion of IL-6 by the peripheral blood mononuclear cells of cachectic pancreatic cancer patients undergoing an ongoing elevated APPR. Thus IL-6 would appear to play a role in the induction of APPR

and elevation of REE which may be the major determinants of the development of cachexia in these patient groups.

However, whilst Tanaka *et al* (1993) reported an elevation of serum IL-6 in mice bearing the colon-26 adenocarcinoma, they were unable to determine whether the observed alterations in host cytokines were a consequence, or a cause, of cachexia. Additionally, Fujimoto-Ouchi *et al* (1995), concluded that although IL-6 was elevated in the serum of mice bearing the cachexia-inducing clone 20 of colon-26, an elevation which could be attenuated by the administration of anti-IL-6 MAb, serum IL-6 levels were also increased in those mice bearing the non-cachexigenic clone 5 of this tumour. Furthermore, Soda *et al* (1994) detected circulating levels of IL-6 in mice bearing clone 5 of the colon-26 tumour equivalent to those recorded in mice bearing clone 20, and so concluded that IL-6 was not the sole inducer of the cachectic state in this model. Similarly, Mulligan *et al* (1992b) were unable to detect elevated circulating levels of IL-6 in mice bearing the MAC16 colon adenocarcinoma, thus precluding IL-6 from any involvement in the development of cachexia in this tumour model. Additionally, massive doses of recombinant human IL-6 (rh IL-6) were required to induce a cachectic syndrome which was similar, but not identical, to that observed during tumour-induced cachexia (Soda *et al*, 1995).

Thus whilst IL-6 may play an important role in the orchestration of a whole host of cytokines or exogenous factors responsible for the development of cachexia, it seems unlikely that IL-6 alone is responsible for this profound catabolic state. Elevated serum IL-6 levels may be associated with poor prognosis and more rapid progression of disease in cancer patients, however, there is no consistent evidence for the involvement of IL-6 in the induction or advancement of host wasting in animal models of cachexia.

1.2.3.4 Ciliary Neurotrophic Factor (CNTF)

Ciliary neurotrophic factor (CNTF), a member of the IL-6 superfamily, is a 23kDa cytoplasmic protein which is found predominantly in glial cells, particularly Schwann cells

of the peripheral nervous system, where it is known to promote the survival of a variety of neuronal and non-neuronal cells (Henderson *et al*, 1996). Mice implanted with C6 glioma cells modified to secrete CNTF demonstrated rapid catabolism of adipose tissue and skeletal muscle, without the development of anorexia, which was accompanied by significant hypoglycaemia and hypotriglyceridaemia. Such profound effects were not observed in mice transplanted with unmodified C6 cells (Henderson *et al*, 1996). Subcutaneous administration of CNTF induced anorexia in recipient mice concomitant with increased muscle catabolism and elevated hepatic acute phase protein production (Espat *et al*, 1996). Thus, it would appear that whilst CNTF has not yet been implicated in the pathogenesis of cancer cachexia, its metabolic effects are such that further investigations are required in order to ascertain whether or not this cytokine could play a role in host catabolism.

1.2.3.5 Leukaemia Inhibitory Factor (LIF)

The human melanoma cell line SEKI has been demonstrated to induce severe, progressive cachexia in nude mice (Mori *et al*, 1989). Analysis of conditioned medium from the SEKI cell line revealed the presence of LIF, a 40kDa protein capable of inhibiting LPL activity (Mori *et al*, 1989), and thus inducing hypertriglyceridaemia and adipose tissue catabolism in recipient animals. No cross-reactivity between LIF and anti-TNF- α antibodies could be demonstrated (Kawakami *et al*, 1991), and so the cachexia-inducing effects of the SEKI cell line appear to occur solely due to its secretion of LIF. Northern blot analysis of the mRNA content of four different human melanoma cell lines revealed that only two (SEKI and G361) expressed LIF mRNA, whilst two other cell lines (A375 and MEWO) did not (Mori *et al*, 1991). A neuroepithelioma cell line (NAGAI) was also found to contain a large amount of LIF mRNA, which could be related to the production of large amounts of LIF protein both *in vivo*, and *in vitro* (Iseki *et al*, 1995).

The mode of inhibition of LPL activity by LIF appears to be very similar to that of TNF- α , with TNF- α demonstrating a greater capacity to inhibit LPL activity and LPL mRNA (Berg *et al*, 1994) and a synergistic reduction in LPL activity occurring when LIF and

TNF- α are combined (Berg *et al*, 1994). However, whilst TNF- α has been shown to reduce lipogenesis, via a downregulation of the expression of the mRNA for acetyl CoA carboxylase (the rate limiting enzyme of lipogenesis), LIF increases acetyl CoA carboxylase mRNA and thus lipogenesis (Marshall *et al*, 1994). Interestingly, Berg *et al* (1994) reported that unlike TNF- α , LIF did not downregulate LPL mRNA, and so proposed that LIF inhibited LPL activity primarily through effects on post-transcriptional processing, conversely, Marshall *et al* (1994) discovered a reduction in LPL mRNA which paralleled the observed reduction in LPL activity, and thus postulated that the effects of LIF on LPL activity occur predominantly via transcriptional regulation.

Therefore, whilst LIF would appear to be implicated in the progression of host wasting in certain human-derived tumours, its exact mechanisms of action in the suppression of LPL activity remain unclear. Additionally, although LIF is known to be capable of inducing the reduction of host adipose tissue observed in cachectic individuals, it has not been conclusively demonstrated to exert catabolic actions upon skeletal muscle. Likewise, there is, as yet, no evidence that the administration of anti-LIF antibodies to nude mice bearing LIF-producing tumours would attenuate the development and progression of cachexia, nor have the circulatory levels of LIF present in cachectic cancer patients been evaluated.

1.2.3.6 Interferon- γ (IFN- γ)

A study into the cachexia observed in mice bearing the Lewis lung carcinoma has suggested the involvement of IFN- γ in both the promotion of tumour cell proliferation, and the induction of progressive host body weight loss (Matthys *et al*, 1991a). Administration of anti-IFN- γ monoclonal antibodies to Lewis lung tumour-bearing mice markedly attenuated the development of host wasting, thus implicating IFN- γ , either directly or indirectly, in the mediation of cachexia in this tumour model. Inoculation of nude mice with genetically modified, IFN- γ -secreting CHO cells further confirmed the ability of IFN- γ to induce a state of cachexia in recipient animals (Matthys *et al*, 1991b). Furthermore, the development

of cachexia in this experimental tumour model could be averted via the pre-treatment of animals with anti-IFN- γ MAb.

However, whilst Matthys *et al* (1991b) were able to detect IFN- γ in the circulation of nude mice inoculated with the genetically modified CHO cell line, they were unable to detect IFN- γ in the serum of C57BL/6N Lewis lung tumour-bearing mice (Matthys *et al*, 1991a). Moreover, whilst IFN- γ is known to affect lipogenesis and lipolysis in cells *in vitro*, there have been no studies reporting its effects on skeletal muscle, nor have the levels of circulating IFN- γ present in the serum of cachectic cancer patients been documented. Therefore, whilst it may appear probable that IFN- γ may mediate cachexia via its role in the release of other cytokines from cells of the immune system, there is, as yet, no consistent evidence for an elevation of IFN- γ levels in progressive host wasting.

1.2.3.7 Proteolysis Inducing Factors (PIF)

The important prognostic significance of muscle wasting in cachectic cancer patients has produced a vast field of research dedicated to the elucidation of the exact mechanisms responsible for this phenomenon. Whilst a reduction in protein synthesis has been cited by some workers (Rennie *et al*, 1983) as the dominant characteristic of muscle wasting, it has also been postulated that an enhanced proteolytic rate, and hence increased muscle protein degradation may be the principal mechanism (Tessitore *et al*, 1987), whereas, increased rates of proteolysis allied with a depression of the protein synthetic rate have been implicated in other experimental models (Smith and Tisdale, 1993a). The possible role of novel tumour-derived proteolysis inducing factors (PIF) in the progression of muscle wasting in cachexia has been widely investigated.

An accelerated rate of proteolysis was observed when the serum of patients undergoing trauma or sepsis was added to rat skeletal muscle preparations *in vitro*, and was postulated to occur due to the presence of a 4274Da, 33 amino acid, glycopeptide in patient plasma (Clowes *et al*, 1983). However, it was also possible to isolate this factor from the serum of

fasted control subjects, and so its presence could merely be the consequence of the normal adaptive response to conditions of semi-starvation. Curiously, whilst Mitchell and Norton (1989) failed to observe any elevation in the rate of protein degradation in rat muscles exposed to cancer patient plasma, Belizario *et al* (1991) documented the isolation of a biologically active factor capable of enhancing rat skeletal muscle protein catabolism *in vitro* from the plasma of weight-losing cancer patients, and the conditioned medium of activated macrophages.

A circulatory PIF was also found to be present in the serum of cachectic mice bearing the MAC16 colon adenocarcinoma (Smith and Tisdale, 1993a), with the greatest rate of proteolytic activity being manifest in serum from mice who had lost 11-20% of their original body weight (Smith and Tisdale, 1993b). Subsequent isolation of the MAC16-derived PIF, from either MAC16 tumours or cultured MAC16 cells, revealed it to be a 24kDa proteoglycan (p24) which exhibited no homology to any of the known cytokines or other eukaryotic proteins (Todorov *et al*, 1996a). Further functional and immunological studies disclosed evidence that the biological activity of p24 is mediated via the *N*- and *O*-linked oligosaccharide chains present in the molecule, and that p24 is in fact a sulphated glycoprotein (Todorov *et al*, 1997). The 24kDa PIF was also found to be present in the urine of cachectic pancreatic cancer patients losing >1.5kg/month, but not in patients losing <1.3kg/month nor patients with burns, major trauma, sepsis, surgical catabolism or normal subjects (Todorov *et al*, 1996a). When administered intravenously to non tumour-bearing NMRI mice p24 induced a state of catabolism, characterised by a decreased rate of protein synthesis, increased muscle degradation and an elevation of PGE₂ (Cariuk *et al*, 1997), resulting in a 29% loss of host adipose tissue, and a 14% loss of carcass dry weight (Todorov *et al*, 1996a), which could be prevented by the prior administration of a MAb derived from hybridomas produced by the fusion of mouse myeloma cells with splenocytes from non-cachectic MAC16 tumour-bearing mice (Todorov *et al*, 1996b). The 24kDa PIF was further demonstrated to induce the breakdown of skeletal muscle fibres via an activation of the ATP-ubiquitin-dependent pathway (MJ Lorite, personal communication).

Thus it would appear that the presence of p24 is specific to cancer associated cachexia, and that this sulphated glycoprotein is responsible for the profound wasting of host skeletal muscle mass observed in certain advanced cancer patients, but is not allied with the cachexia observed in burns or trauma patients. Further investigations are now required in order to determine whether p24 is solely capable of inducing protein degradation or whether it may also be capable of attenuating protein synthesis, thus accelerating host muscle wasting, and to fully elucidate the signal transduction pathways associated with PIF, assisting the development of therapeutic antagonists.

1.2.3.8 Lipid-Mobilising Factors (LMF)

The existence of a factor with lipid-mobilising activity in the urine of cancer patients was initially reported in 1958 by Chalmers *et al*, who also detected an identical factor in the urine of fasted (36h), but not fed, normal subjects. Sub-cutaneous administration of an extract purified from the urine of starved subjects to non tumour-bearing mice, resulted in an increased mobilisation of adipose tissue stores accompanied by an increased total metabolic turnover of fat, producing a reduction of host body weight without the induction of anorexia.

Numerous lipid-mobilising factors have subsequently been isolated from the serum, ascites fluid and urine of cachectic cancer patients. The significant decrease in carcass neutral lipids observed in rats bearing the Walker 256 carcinoma lead Kralovic *et al* (1977) to postulate the involvement of a tumour-derived lipolytic factor, which they found to be present in the ascites, but not blood serum, of tumour-bearing animals. Likewise, Theologides (1976) detected the presence of both anorexigenic and lipolytic peptides in the urine of patients with widespread neoplastic disease. Thompson *et al* (1981) observed a decrease in adipose tissue LPL activity accompanied by increased basal lipolysis in mice bearing the preputial gland tumour ESR-586, at low tumour burdens, and postulated that such observations may occur as a consequence of the production of humoral factors by the growing tumour.

Similarly, Kitada *et al* (1980) documented the isolation of a lipid-mobilising factor from the serum of non-anorectic, cachectic tumour-bearing mice which induced a profound mobilisation of adipose tissue stores when administered to non tumour-bearing mice. An identical heat stable, 5kDa, proteinaceous factor was also found to be present in the serum of a patient with advanced cancer, and could be isolated from the culture medium of a human lymphoma cell line (Kitada *et al*, 1981), thus implying this factor to be a direct product of the tumour cells rather than the result of the host's response to the presence of a tumour. Further studies, employing a thymic lymphoma transplanted into AKR mice, revealed a tumour-derived factor which: was relatively heat stable; sensitive to trypsin treatment; possessed a molecular weight of less than 10kDa; was not a lipase and which became lipolytically active following storage at 4°C for 4-5 days (Kitada *et al*, 1982). Kitada *et al* (1982) thus postulated that as this factor was not found to be present in the serum of healthy subjects, it must be a direct product of the lymphoma cells, and that it induced lipolysis in isolated adipocytes via a receptor mediated mechanism in a similar fashion to the catecholamines.

Masuno *et al* (1981) described the isolation of a lipolytic substance from the ascites fluid of Sarcoma 180-bearing mice, which was visualised as a single 75kDa band on SDS PAGE following ammonium sulphate fractionation. This heat stable protein, which they termed toxohormone-L, could also be detected in patients bearing hepatomas or Grawitz's tumour, and was believed to be responsible for the reduced host adipose tissue and increased plasma FFA observed in these patients. Masuno *et al* (1984) further reported the purification of toxohormone-L from the ascites fluid of hepatoma, lymphoma and ovarian cancer patients and demonstrated that injection of toxohormone-L into the lateral ventricle of the rat brain significantly reduced both food and water intake whilst stimulating adipose tissue lipolysis. The appetite suppressing actions of toxohormone-L were also noted by Okabe *et al* (1992), who postulated that the direct anorexigenic effects of toxohormone-L on the VMH may result in the induction of anorexia in cancer patients. Thus the lipid depleting mechanisms

of this factor would appear to occur more as the consequence of its ability to induce a state of semi-starvation rather than any direct lipid-mobilising actions.

A heat stable, 6kDa, glutamic acid rich peptide was isolated from the extracellular environment of cultured human tumour cells and discovered to induce lipolysis in isolated adipocytes in both a time- and dose-dependent manner, and could be correlated to the *in vivo* loss of body lipids in tumour-bearing animals (Taylor *et al*, 1992). This factor, designated lipolysis promoting factor (LPF), was also demonstrated to be present in both the serum and ascites fluid of ovarian cancer patients, and to be associated with an increased expression of hormone-sensitive lipase (HSL) in normal adipocytes (Gercel-Taylor *et al*, 1996).

The MAC16 adenocarcinoma was also reported to produce a factor with the ability to induce lipolysis in isolated adipocytes via an activation of the signal transduction pathway classically associated with adrenergic receptor agonists. Incubation of a single cell suspension of murine white adipocytes with crude MAC16 tumour supernatants revealed an enhanced, linear, time-dependent release of FFA (Beck and Tisdale, 1987). Conversely, tumour supernatants prepared from two closely related, non-cachexigenic colon adenocarcinomas, MAC13 and MAC15A, demonstrated less than one third of the lipolytic activity observed with MAC16. The LMF was likewise detected in the urine and serum of patients with clinical cancer cachexia, but was not present in normal sera, even in conditions of starvation (Beck *et al*, 1990a; Groundwater *et al*, 1990). Intraperitoneal administration of LMF to both MAC13 tumour-bearing mice (Beck *et al*, 1990a), and non tumour-bearing mice (Cariuk *et al*, unpublished results), induced a dose-dependent decrease in host body weight due to a preferential loss of host body fat, without any alterations of either total body water or lean body mass. Chromatographic purification of LMF produced four peaks of lipolytic activity; >20kDa, 3kDa, 1.5kDa and <0.7kDa (Beck and Tisdale, 1991), whilst further purification and characterisation studies revealed LMF to be a protein with an apparent molecular weight of 40kDa which demonstrated sequence

homology and identical gel migratory and antibody reactivity properties as Zn- α -2-glycoprotein (Zn α ₂gp) (McDevitt, 1996).

The purification of Zn α ₂gp from both normal human plasma (Burgi and Schmid, 1961) and human seminal plasma (Ohkubo *et al.*, 1990) has been documented, together with suggestions that their post-translational modifications may vary. Enhanced expression of Zn α ₂gp cDNA has been reported in women with benign breast lesions, whilst variable results have been noted in malignant breast tumours (Freije *et al.*, 1991), thus it would appear that there may be a role for Zn α ₂gp in the pathology of certain disease states. Additionally, the homology observed to exist between Zn α ₂gp and class I histocompatibility antigens has led to the postulation that Zn α ₂gp may play a role in the expression of the immune response (Araki *et al.*, 1988), and so could possibly modulate the production of cytokines, and thus the manifestation of cachexia in the tumour-bearing host. The expression of a high level of Zn α ₂gp mRNA could be detected in the MAC16 tumour (McDevitt *et al.*, unpublished results), a finding which, allied with the sequence homology existing between Zn α ₂gp and LMF, could imply that Zn α ₂gp may be involved in the induction of cachexia by the MAC16 tumour.

So far, however, the exact biological role of Zn α ₂gp remains unknown, and there has been no conclusive evidence that Zn α ₂gp itself is responsible for the induction of cachexia in tumour-bearing animals or cancer patients. It is possible that Zn α ₂gp may possess the ability to activate adenylate cyclase (AC) and thus induce lipolysis in adipose tissue. However, it is also possible that Zn α ₂gp may be acting merely as a carrier protein, or antigen presenting molecule, transporting either a conventional lipolytic hormone, some novel tumour-produced adrenergic analogue or even a tumour antigen with the ability to induce the production of lipolysis-inducing host cytokines. Therefore, whilst Zn α ₂gp may appear a likely candidate for the identification of MAC16 tumour-derived LMF, it cannot be categorically stated to be the factor responsible for the depletion of lipid stores observed in

cancer cachexia until recombinantly produced $Zn\alpha_2gp$ has been demonstrated to possess direct lipolytic actions *in vitro*, and the ability to mobilise adipose tissue deposits *in vivo*.

1.2.3.9 Other Putative Humoral Mediators of Cachexia

Several other factors have been postulated as possible mediators of the cachectic state. In a study of stage IV breast cancer patients Knapp *et al* (1991) reported significantly elevated fasting plasma levels of glucagon and serum cortisol in patients with weight loss, and suggested that these factors may mediate the development of cachexia. Sakayama *et al* (1994) examined several human sarcomas and carcinomas and discovered a universally elevated expression of active 57kDa LPL, thus overexpression of LPL may itself be sufficient to induce cachexia. Interestingly, Bartlett *et al* (1995) noted an elevation of circulating levels of glucagon in rats bearing MAC33 mammary adenocarcinomas, and proposed that a reduced insulin/glucagon ratio may be the causative factor of cachexia in this tumour model. A tumour-induced up-regulation of circulating levels of adrenaline, noradrenaline and corticosterone was postulated to be responsible for the significantly increased basal lipolysis, hypertriglyceridaemia and reduced epididymal fat pad size observed in mice bearing Ehrlich ascites tumours (Balint, 1991). Furthermore, transforming growth factor $\beta 1$ (TGF $\beta 1$) has been demonstrated to possess systemic effects, including the ability to induce cachexia (Zugmaier *et al*, 1991) whilst Matzuk *et al* (1994) postulated that a deficiency of the growth regulatory factor inhibin (a member of the TGF β family) may result in the development of adrenal tumours and a cancer cachexia-like syndrome in mice.

In conclusion, many disparate humoral mediators of the cachectic process have been proposed. The relative importance of some of these factors is likely to differ, depending largely upon tumour type, with some tumours producing novel catabolic factors whilst others may possess the ability to induce cachexia via modulation of the host's own cytokine network. A whole range of novel tumour-derived factors with both proteolytic and lipolytic activities have been documented, and fuller characterisation, allied with isolation of the

genes responsible for their production and thus elaboration of pure recombinant factors, should enable further studies to reveal whether any of these mediators is ubiquitously expressed by all cachexigenic tumours, or whether each tumour type has its own unique mechanism for the induction of host wasting.

1.2.4 Therapeutic Approaches to Cancer Cachexia

Customarily, the principal therapeutic aim in the treatment of cancer is to attack the tumour, with minimal attempt being made to alleviate the patient's symptoms (Walsh, 1994). Whilst resection of the tumour, and thus removal of the cause of host wasting, would appear to be the ideal treatment for cachexia (Fearon *et al*, 1988) this is rarely feasible. Additionally, whilst almost all chemotherapeutic agents adversely affect dietary intake, poor host nutritional status has been correlated to indifferent responses to anti-neoplastic therapy (Donaldson and Lenon, 1979).

A variety of disparate strategies have been employed in the endeavour to overcome the severe wasting observed in certain cancer patients, and thus increase the probability of a favourable response to other anti-neoplastic therapies. Whilst some studies have aimed to correct the patients reduced nutritional status via an increased caloric intake, others have attempted to reduce the elevation of REE which has frequently been observed. In addition drugs capable of either stimulating appetite, or directly counteracting the catabolic effects of a number of the postulated humoral mediators of cachexia have also been utilised in an attempt to arrest, if not reverse, the loss of skeletal muscle and adipose tissue mass experienced by these patients.

1.2.4.1 Hyperalimentation

The administration of additional calories to cachectic cancer patients would seem the obvious means of correcting any reduction in voluntary caloric intake, and could be of value as a means of preserving, or correcting, the nutritional status of the host and hence preventing further weight loss. Earlier diagnosis allied with improved supportive care may

substantially increase patient survival, markedly improve quality of life, and enhance responsiveness to other anti-neoplastic therapies (Walsh, 1994). Several different approaches to increasing caloric intake may be employed in cancer patients. Based on the patient's baseline deficit, and the nutritional consequences of any cancer therapy, nutritional intervention may range from patient education, advice and encouragement, to the use of enteral tube feeding or parenteral nutrition (Ottery, 1996). It has been suggested that wherever possible enteral and parenteral nutrition should be avoided (Tchekmedyian *et al*, 1992), however, enteral tube feeding may prove beneficial to those patients with disruption of the swallowing mechanism or obstruction of the upper GI tract, whilst parenteral, central venous nutrition may be necessary in those patients whose digestive system is not fully functional (DeWys, 1985; Ottery, 1995).

Terepka and Waterhouse (1956) postulated that the only feasible means of correcting the voluntary decrease in food intake in anorexic, hypermetabolic weight-losing cancer patients was forcible enteral supplementation of dietary intake, employing gastric tube feeding where necessary. Such an aggressive approach, however, merely increased host body weight via alterations in intracellular fluid and fat, not lean body mass. Furthermore, these effects were purely transient, and once supplemental nutrition was discontinued host wasting continued at a greater rate than previously observed, suggesting an acceleration of the malignant process and questioning the benefits of forced feeding. A detrimental acceleration of tumour metabolism upon administration of nutritional support was likewise observed by DeWys (1985).

An increase in adipose tissue mass, without significant increases in host muscle mass was similarly noted in 15 cancer patients given either central venous or enteral hyperalimentation (Nixon *et al*, 1981). Cohn *et al* (1982) also recorded a minimal increase in host lean body mass (0.3-0.6kg) following total parenteral nutrition (TPN) with larger gains of total body water and fat, thus implying that whilst cancer may not impede lipogenesis it does attenuate the ability of the host to utilise nutrients for the synthesis of lean body mass. In addition,

Torosian and Daly (1986) concluded that neither tumour growth, host toxicity or tumour response to therapy could be manipulated by nutritional therapy. Interestingly, Hyltander *et al* (1991) reported that the administration of a higher calorie TPN (equivalent to 200% of REE) may beneficially influence whole body protein synthesis rates. However, this improvement in lean body mass was accompanied by a concomitant increase in REE, thus implying that TPN may not markedly enhance the patient's nutritional status long term, but rather exacerbate some of the manifestations of cachexia.

The predominant intention of nutritional support should be an improvement in the quality of life experienced by the patient, and, where possible, a stabilisation or amelioration of nutritional status (Tchekmedyian *et al*, 1992). However, simply forcing the patient to ingest an increased number of calories may not be sufficient to rectify the deficit in nutritional status. Whilst increasing dietary intake may result in a significant gain in body fat and lean muscle mass in patients with anorexia nervosa (Orphanidou *et al*, 1997), the complex metabolic alterations induced by the tumour will influence weight gain after nutritional intervention, resulting in a disproportionate gain of fat and water without a significant increase in lean body mass. Furthermore, DeWys (1985) noted that whilst nutritional support alone may not be beneficial, the concomitant administration of extra calories with anticancer therapy may prove to be of positive benefit to the patient. Indeed there have even been suggestions that, where possible, enteral and parenteral nutrition should be avoided (Tchekmedyian *et al*, 1992) and other therapeutic approaches to cachexia, such as appetite stimulating drugs, employed.

1.2.4.2 Megestrol Acetate

Megestrol acetate is a synthetic, orally active, derivative of the hormone progesterone which is principally employed in the treatment of non-resectable, oestrogen-dependent breast carcinoma at a recommended daily dose of 160mg (Strang, 1997). The observation of weight gain in patients treated with megestrol acetate resulted in the systematic study of this progestin as a therapy for cachexia in both cancer patients (Loprinzi *et al*, 1992),

experimental tumour models (Beck and Tisdale, 1990) and AIDS patients (Von Roenn *et al*, 1992; Strang, 1997).

In a study of 133 cachectic cancer patients Loprinzi *et al* (1992) recorded an increased appetite, accompanied by a suppression of nausea and emesis, and the promotion of non-fluid weight gain, after the administration of 800mg/day megestrol acetate. Likewise, Heckmayr and Gatzemeier (1992) noted significant weight gain, allied with minimal oedema, in cachectic lung cancer patients given 160mg/day or 480mg/day megestrol acetate. Similarly, a dose of 160mg twice daily improved appetite and performance status, and induced non-fluid weight gain in a group of 24 patients with advanced bladder cancer (Neri *et al*, 1995). Additionally, in a Phase III evaluation of four different doses of megestrol acetate (160, 480, 800 or 1280mg/day) in 342 cachectic cancer patients Loprinzi *et al* (1994) demonstrated a trend for a greater degree of non-fluid weight gain with higher drug doses, and concluded the optimal dose of megestrol acetate, which produced the best improvement in appetite and food intake, to be 800mg/day. However, administration of megestrol acetate at such doses may prove expensive, costing between \$15-20/day (Loprinzi *et al*, 1992), and so there is a tendency to recommend the use of lower initial doses of this drug (160mg/day), escalating dosage to 800mg/day only when necessary (Loprinzi *et al*, 1994).

Studies into the alterations in body composition induced by administration of megestrol acetate revealed that whilst the development of cachexia in MAC16 tumour-bearing mice was effectively attenuated by megestrol acetate, weight gain was principally the result of oedema and increased body fat (Beck and Tisdale, 1990), implying that whilst megestrol acetate may prevent, or rectify, body wasting, it does not prevent the loss of host muscle mass. Likewise, Loprinzi *et al* (1994) and Strang (1997) also noted that in cancer patients the majority of weight gained due to megestrol acetate therapy was the consequence of an expansion of host adipose tissue stores, and not muscle mass.

Therefore, whilst megestrol acetate may have demonstrable benefits, preventing host weight loss and even inducing weight gain, it would appear that these occur as a consequence of increased lipid storage, and not as the result of a complete reversal of the cachectic process. Thus, despite favourable outcome in some patients, it would appear that both the expense of this drug, and its inability to replace lost muscle mass, the major prognostic factor in host wasting, should preclude megestrol acetate from being regarded as the sole optimal therapeutic strategy for the treatment of cancer cachexia.

1.2.4.2.1 Other Appetite Stimulating Drugs

Various pharmacological approaches have been adopted in an attempt to stimulate appetite, and thus counteract anorexia in weight-losing cancer patients. The most widely investigated appetite stimulating drug is megestrol acetate, but in addition: the methyl xanthine-derivative pentoxifylline; the marijuana-derivative dronabinol; the anti-serotonergic anti-histamine cyproheptadine; corticosteroids such as prednisolone, methylprednisolone or dexamethasone; and the gluconeogenesis inhibitor hydrazine sulphate have all been postulated to increase voluntary dietary intake (Chlebowski, 1991; Bruera, 1992; Tchekmedyian *et al*, 1992; Ottery, 1995).

Oral administration of pentoxifylline to a heterogeneous group of cachectic cancer patients failed to attenuate body weight loss (Goldberg *et al*, 1995). Likewise, cyproheptadine, which has been reported to stimulate appetite via an alteration of the concentration of serotonin present in the CNS (Ottery, 1995), did not induce any confirmed increase in caloric intake in cachectic cancer patients (Tchekmedyian *et al*, 1992). In contrast, dronabinol (5mg/day) significantly reduced the rate of body weight loss, whilst improving appetite and mood (Bruera, 1992), however, any possible long-term side effects of this cannabinoid remain to be ascertained.

Short-term, corticosteroids have been demonstrated to beneficially improve both appetite and well-being (Chlebowski, 1991), such benefits are, however, transient and long-term

administration of drugs such as prednisolone can result in reduced efficacy allied with increased side effects (Tchekmedyan *et al*, 1992). Clinical trials employing hydrazine sulphate have also failed to establish consistent evidence of any improvement in the cachectic state (Tchekmedyan *et al*, 1992), despite evidence of increased median survival when administered in combination with conventional chemotherapy (Bruera, 1992).

Thus, attempts to increase voluntary caloric intake via the administration of drugs which have been demonstrated to increase appetite in other disease states, have failed to provide conclusive evidence of beneficial effects in weight-losing advanced cancer patients. It would appear, therefore, that further investigations are required in order to identify additional orexigenic drugs which may prove to possess a greater efficacy to combat anorexia in cachectic cancer patients.

1.2.4.3 Anti-Cytokine Therapies

A number of varied therapeutic regimens have been employed in an attempt to attenuate the production of pro-inflammatory cytokines, and thus prevent the development of cancer cachexia. Early treatment of mice bearing the Lewis lung carcinoma, a tumour which induces profound wasting of host lipid stores, with anti-IFN- γ MAb inhibited tumour growth, whilst both early and late administration of anti-IFN- γ prevented wasting of host adipose tissue, thus preventing the development of cachexia in this experimental model (Matthys *et al*, 1991a).

The methylxanthine derivative, pentoxifylline has been demonstrated to reduce TNF- α mRNA expression, and TNF- α production, and hence prevent the progression of TNF- α -dependent cachexia (Ottery, 1995). However, administration of pentoxifylline (400mg 3 times/day) to 70 cancer patients with weight loss, failed to induce host weight gain in this heterogeneous group (Goldberg *et al*, 1995), thus suggesting that TNF- α does not play a major role in the pathology of many cancers, and so anti-TNF- α therapy is not likely to produce significant clinical benefits. The pineal hormone melatonin (MLT) has also been postulated as an anti-cachectic agent, due to its ability to inhibit TNF- α secretion (Lissoni

et al, 1996). Administration of 20mg/day oral MLT, together with supportive care, to patients with untreatable, metastatic, solid tumours did not alter food intake. However, the patient group receiving MLT demonstrated a reduced rate of weight loss when compared to similar patients receiving supportive care alone, thus suggesting that MLT may be effective in treating cachexia via a suppression of serum TNF- α levels.

Intratumoural injections of an IL-1 receptor antagonist (IL-1RA) whilst having no effect upon tumour burden, have been found to prevent the catabolism of adipose tissue and muscle, and the hypoglycaemia associated with IL-1 administration (Strassman *et al*, 1993). In addition, IL-1RA and anti-IL-1 MAb significantly reduced tumour production of IL-6 and attenuated the development of cachexia in mice bearing the colon 26 adenocarcinoma (Strassman *et al*, 1993; Yasumoto *et al*, 1995).

Furthermore, Strassman *et al* (1992) demonstrated that administration of anti-IL-6 MAb to colon 26 tumour-bearing mice also resulted in increased body weight and epididymal fat pad size, without affecting tumour burden, whilst the polyunsaturated fatty acid (PUFA) eicosapentaenoic acid (EPA) has been suggested to down-regulate APPR in cachectic pancreatic cancer patients via a suppression of IL-6 (Wigmore *et al*, 1997c). Similarly, the transfection of the gene for IL-10 into the cachexigenic, IL-6 secreting clone 20 of colon 26 resulted in increased circulatory levels of IL-10 which correlated with decreased serum IL-6, and reduced expression of IL-6 mRNA at the tumour site, accompanied by an attenuation of host wasting (Fujiki *et al*, 1997).

In conclusion, the utilisation of either drugs which antagonise cytokine production, or anti-cytokine MAb may prove to be an efficacious approach for the treatment of cachexia in those patients who manifest elevated circulating concentrations of pro-inflammatory cytokines. Such a therapeutic strategy may be expected to inhibit the end organ effects of the cytokine(s) in question, thus preventing both the direct catabolism of host muscle and adipose tissue, and the occurrence of an APPR, hence averting any elevation of REE.

However, the use of anti-cytokine therapies will be of little benefit in those patients demonstrating non cytokine-dependent cachexia. Therefore, treatments designed to antagonise the actions of pro-inflammatory cytokines should not be regarded as universally effective in all weight-losing cancer patients, only those with a demonstrable increase of serum cytokine levels.

1.2.4.4 Anti-Inflammatory Drugs

The non-steroidal anti-inflammatory drug (NSAID) ibuprofen, has been demonstrated to inhibit the cyclo-oxygenase (COX) pathways of prostaglandin production, thus attenuating APPR in cachectic pancreatic (Wigmore *et al*, 1995), and colonic (Preston *et al*, 1995), cancer patients. At a dose rate of 400mg 3 times per day, ibuprofen reduced serum CRP levels in both patient groups, and attenuated the elevated REE observed in the pancreatic cancer patient group.

Similarly, another COX inhibitor, indomethacin, prolonged survival, reduced tumour growth and increased food intake and motor activity in cachectic, anorectic rats bearing a methylcholanthrene-induced sarcoma when administered at a dose rate of 1mg/kg/day (Sandstrom *et al*, 1990). Indomethacin (1µg/g body weight) also prolonged survival, reduced tumour growth and attenuated cachexia in mice bearing the PGE₂ secreting malignant melanoma K1735-M2, but was ineffective against a non-prostaglandin producing epithelial-like tumour, MCG101 (Lonnroth *et al*, 1995). Combined administration of indomethacin with a synthetic low-toxicity lipid A derivative, DT-5461a, to mice bearing colon 26 carcinoma resulted in an increased survival time allied with decreased circulating PGE₂ (Jimbo *et al*, 1995).

Thus, the administration of NSAIDs such as ibuprofen or indomethacin to cachectic cancer patients may successfully attenuate the progression of host wasting. Both drugs reduced the APPR which is frequently observed in cachexia, and has been associated with elevated REE (Falconer *et al*, 1994a), via a downregulation of prostaglandin production. Whilst

such drugs may not attenuate cachexia in all advanced cancer patients, it would appear that their administration to hypermetabolic patients may reduce REE hence preventing, or delaying, host wasting.

1.2.4.5 Insulin and Insulin-like Growth Factor-1 (IGF-1)

The observation of abnormal glucose tolerance in cachectic cancer patients, allied with the knowledge that insulin reduced intracellular cAMP in adipocytes, thus inhibiting lipolysis, whilst suppressing proteolysis and promoting the uptake of amino acids into skeletal muscle, prompted Schein *et al* (1979) to postulate a potential role for insulin in the nutritional management of cachexia. In a study employing 24 Fischer rats bearing the MCA sarcoma, Kern and Norton (1987) noted a reversal of insulin resistance accompanied by an increased uptake of both glucose and amino acids by host skeletal muscle upon administration of 0.5U insulin/100g/day. No concomitant elevation of nutrient uptake by the tumour was observed, and hence Kern and Norton concluded that insulin therapy preserved host body weight and muscle mass via a reversal of anorexia and a shift of nutrient flow from the tumour towards the host. In a similar study Brauer *et al* (1994) noted a preservation of host body weight in rats bearing a methylcholanthrene-induced tumour receiving 2U insulin/100g body weight/day.

Similarly, O'Riordain *et al* (1995) demonstrated a beneficial attenuation of acute phase protein production in human hepatocytes upon exposure to insulin and counterregulatory hormones such as dexamethasone, glucagon and adrenaline. Conversely, the administration of insulin or insulin-like growth factor-1 (IGF-1) to colon 26 tumour-bearing mice failed to prevent continued host wasting (Lazarus *et al*, 1996). In addition, whilst Tisdale and Beck (1990) observed an attenuation of the cachexia induced by the MAC16 colon adenocarcinoma upon sub-cutaneous administration of 20U insulin/kg/day, a significant increase in tumour weight was also noted.

It would seem evident, therefore, that great caution should be exercised, and insulin or IGF-1 should not be regarded as the logical means of rectifying the glucose intolerance prevalent during cachexia. Glucose intolerance may arise due to insulin resistance, not deficient insulin secretion, hence administration of exogenous insulin may exacerbate hypoglycaemia. Therefore, whilst the end organ effects of several humoral cachexigenic factors may be inhibited by the provision of insulin, the risk of stimulating tumour growth, allied with the dangers of hypoglycaemic shock should preclude the utilisation of insulin as a therapeutic strategy.

1.2.4.6 Additional Pharmacological Approaches to Cancer Cachexia

Several disparate drugs have been investigated as potential therapeutic agents in cancer cachexia. Anabolic steroids, such as nandrolone propionate have been demonstrated to increase weight in MCG101 sarcoma-bearing cachectic mice (Lyden *et al*, 1995). Such weight gain, however, was solely the product of increased water retention, with only a minor elevation of net protein content, thus the therapeutic benefits of anabolic steroids in cachexia are negligible. Similarly, the glucocorticoid receptor antagonist RU38486 failed to diminish the loss of host body weight and muscle mass observed in rats bearing the fast-growing ascites hepatoma Yoshida AH-130 (Llovera *et al*, 1996).

In contrast, the β_2 anabolic agonist cimaterol, when administered with supplemental nutrition, significantly increased muscle protein in cachectic rats, whilst markedly attenuating tumour burden (Stallion *et al*, 1991). In addition, the combined administration of the somatostatin octreotide with insulin, effectively reversed hyperglucagonaemia and increased muscle mass in cachectic MAC33 mammary adenocarcinoma-bearing rats (Bartlett *et al*, 1995). Similarly, lipoxygenase (LOX) inhibitors such as BWA4C and 2,3,5-trimethyl-6-(3-pyridylmethyl) 1,4-benzoquinone (CV-6504) have been demonstrated to attenuate both tumour growth and the development and progression of cachexia in MAC16 tumour-bearing mice (Hussey *et al*, 1996).

The hypolipidaemic drugs bezafibrate and clofibrate have also been investigated as potential anti-cachectic agents. However, whilst clofibrate demonstrated anti-tumour activity against the Walker 256 carcinosarcoma, and further potentiated the anti-tumour and anti-cachectic effects of hydrazine sulphate (Gold, 1978), bezafibrate stimulated tumour growth and so exercised deleterious effects upon mice bearing the MAC16 adenocarcinoma (Mulligan and Tisdale, 1991b).

Thus, whilst a multitude of drugs have been postulated to possess the ability to reverse cancer cachexia, few have been demonstrated to be wholly beneficial. The efficacy of such agents may, upon further investigation, be found to vary according to tumour type and stage of disease. However, thus far, adrenergic anabolic agonists, such as cimaterol; somatostatins, such as octreotide; and LOX inhibitors, such as CV-6504 would appear to exhibit potential for further clinical evaluation, and may prove to be the origins of new families of more efficacious anti-cachexia therapies.

1.2.4.7 Dietary Manipulation

Conventional nutritional support has been demonstrated to be ineffective in reversing the progression of cancer cachexia, however, alteration of the composition of dietary supplements may prove beneficial. A decreased tumour growth (33%) and increased survival time (32%) has been observed in Yoshida sarcoma-bearing rats receiving physiological doses of branched chain amino acids (BCAA) such as leucine, isoleucine and valine (Schaur *et al*, 1980). The BCAA diet did not significantly suppress muscle proteolysis, despite promoting the synthesis of carcass proteins, in both tumour-bearing rats (Schaur *et al*, 1980) and cancer patients (Hunter *et al*, 1989). Interestingly, there have also been suggestions that the administration of BCAA may reduce the availability of the serotonin precursor tryptophan and thus reverse anorexia in cancer patients (Cangiano *et al*, 1996a; Cangiano *et al*, 1996b). Conversely, the administration of ornithine α -ketoglutarate did not reverse tumour growth, or tumour-induced host catabolism, in untreated Morris hepatoma 7777-bearing rats (Le Bricon *et al*, 1995).

The provision of the non-nitrogenous calories present in TPN as fat, rather than carbohydrate, has been suggested to promote host maintenance without a concomitant stimulation of tumour growth (Buzby *et al*, 1980), by reducing the availability of glucose for tumour metabolism, whilst increasing the provision of alternative metabolic fuels, such as ketone bodies, to the host. A ketogenic diet, providing 70% of calories as medium chain triglycerides (MCT) has also been demonstrated to induce weight gain and increase body protein mass in cachectic cancer patients, whilst decreasing blood glucose, lactate and pyruvate concentrations (Fearon *et al*, 1988). Furthermore, feeding an isocaloric isonitrogenous diet containing 80% of calories as MCT significantly elevated plasma levels of ketone bodies in MAC16 tumour-bearing mice, whilst restoring nitrogen balance to that seen in the non tumour-bearing state and attenuating both tumour burden and host weight loss (Beck and Tisdale, 1989). Additional dietary modification, employing n-3 fatty acids as the source of dietary lipids, has also been demonstrated to prevent the progression of cachexia in this tumour model (Tisdale and Dhesi, 1990).

Thus, it would appear that nutritional and therapeutic benefits may be obtained via a modification of the dietary intake of the weight-losing cancer patient. Reducing the carbohydrate content of the diet decreases the availability of glucose for tumour metabolism and induces a state of ketosis in the host. Whilst host tissues possess enzymes permitting the utilisation of ketone bodies as a metabolic fuel, such enzymes have been demonstrated to be absent in neoplastic tissues (Tisdale and Brennan, 1983). Therefore, the induction of systemic ketosis, may prove detrimental to the tumour, hence inhibiting tumour growth and the progression of the cachectic state, but beneficial to the host.

1.2.4.8 Polyunsaturated Fatty Acids

The essential fatty acids (EFAs) represent the two most important families of PUFAs in the body. Whilst the majority of the body's fatty acid requirements may be met by *de novo* lipogenesis, the precursors of the n-3 and n-6 EFAs must be taken in the diet. The parent FAs of these two EFA families (18:2, n-6 linoleic acid and 18:3, n-3 α -linolenic acid), can

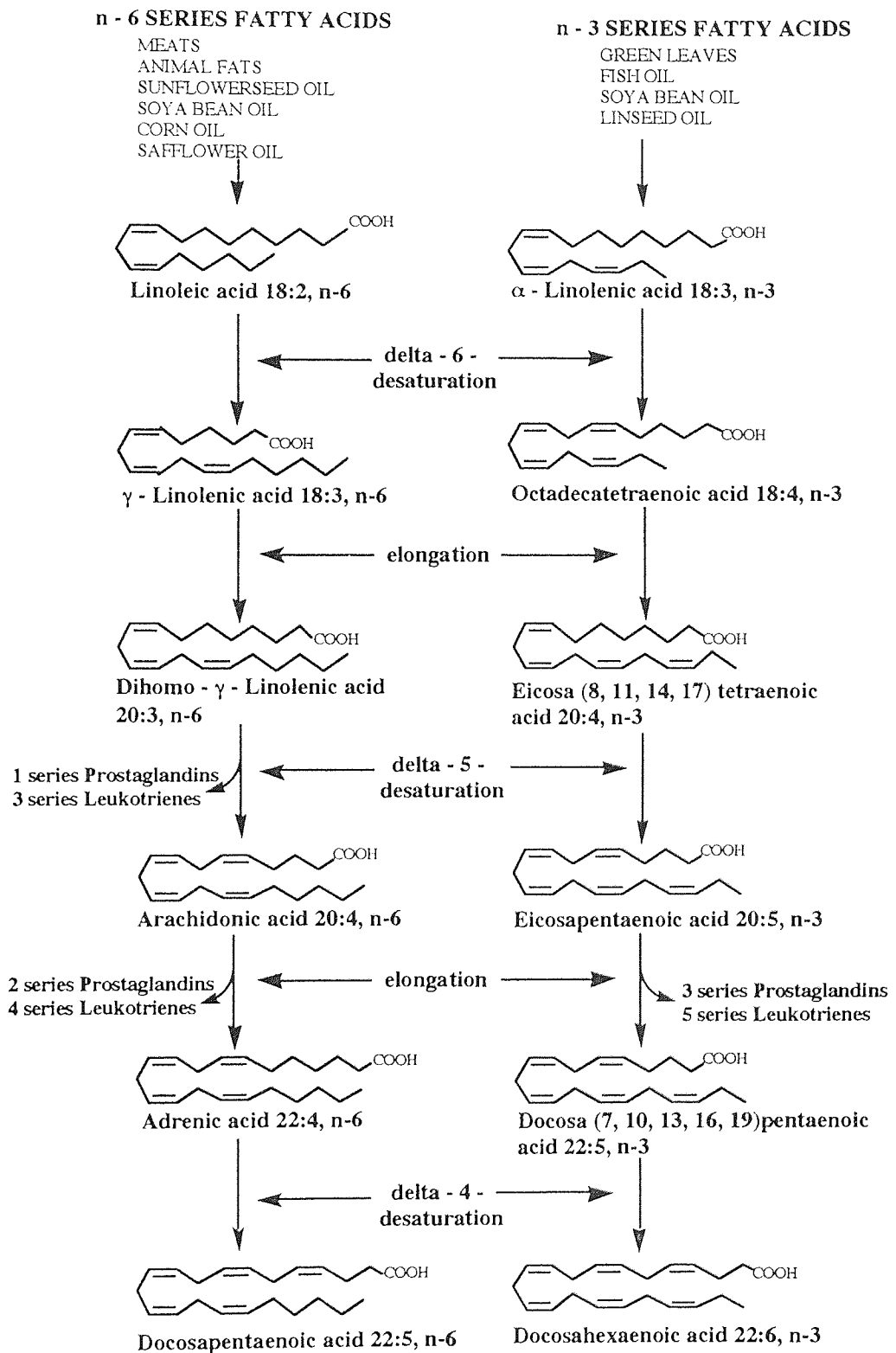


Figure 1.2:- Elongation and Desaturation of the Essential Fatty Acids

however be converted within the body via a series of desaturating and elongating enzymes to produce longer chain, more highly unsaturated PUFAs such as: 20:4, n-6 arachidonic acid (AA); 20:5, n-3 eicosapentaenoic acid (EPA) and 22:6, n-3 docosahexaenoic acid (DHA) (Figure 1.2) (Horrobin, 1994).

The EFAs fulfil many roles within the body, being required to maintain the integrity of cell membranes, mitochondrial membranes and nuclear membranes. A whole range of second messengers are also derived from EFAs, including the prostaglandins, leukotrienes and other eicosanoids. More recently, it has been postulated that FAs interact with almost all second messenger systems, modulating the formation of cytokines, and the behaviour of most membrane bound proteins, including receptors, ion channels and ATPases (Horrobin, 1994). Thus a role for EFAs in the attenuation of cachexia may be implied as a consequence of either reduced cytokine production, or an inhibition of the end organ effects of humoral mediators.

A strong correlation has long been recognised to exist between dietary fat intake, and the risk of developing cancer (Caygill *et al*, 1996), though the quality of fat, rather than the exact quantity, may be the genuine risk factor (Pritchard *et al*, 1989). Several studies have revealed that n-6 PUFAs such as linoleic acid (LA) and AA may stimulate the growth, *in vitro*, of: murine colon adenocarcinomas (MAC13 and MAC26) (Hussey and Tisdale, 1994); human breast cancer cell lines (Rose and Connolly, 1989; Bardon *et al*, 1996); and prostate cancer cell lines (Karmali *et al*, 1987; Pandalai *et al*, 1996). In contrast, n-3 PUFAs have been demonstrated to inhibit the growth and proliferation of: promyelocytic leukaemic HL-60 cells (Finstad *et al*, 1994); rat mammary tumours (Jurkowski and Cave Jr, 1985; Minami and Noguchi, 1996); human lung carcinomas (de Bravo *et al*, 1991); tumourigenic kidney epithelial cells (Maehle *et al*, 1995); and human pancreatic cancer cell lines (Falconer *et al*, 1994b).

In addition, n-3 fatty acids have been observed to modulate the production of various pro-inflammatory cytokines, including TNF- α , IL-1 β (Purasiri *et al*, 1994; Endres *et al*, 1995; Caughey *et al*, 1996; Endres, 1996), IFN- γ (Purasiri *et al*, 1994) and IL-6 (Purasiri *et al*, 1994; Wigmore *et al*, 1997c), and to down-regulate the elevated APPR observed in cachectic cancer patients (Wigmore *et al*, 1996a). Furthermore, n-3 PUFAs have been noted to significantly attenuate weight loss in some experimental models of cancer cachexia (Tisdale and Dhesi, 1990; Dagnelie *et al*, 1994; Ohira *et al*, 1996), but not others (Costelli *et al*, 1995) and to correct hyperinsulinaemia and improve adipocyte responsiveness to insulin in insulin-resistant slightly diabetic rats (Luo *et al*, 1996). Additional studies involving individual PUFAs have suggested that whilst LA is totally ineffective at preventing cachexia (Tisdale and Beck, 1991), GLA (Beck *et al*, 1991), DHA (Ohira *et al*, 1996) and EPA (Tisdale and Beck, 1991, Ohira *et al*, 1996) may prove beneficial.

1.2.4.8.1 γ -Linolenic Acid (GLA)

The 18:3, n-6 PUFA γ -linolenic acid (GLA) has been demonstrated to: competitively inhibit the activity of urokinase, an important protease involved in the invasion and metastasis of malignant cells (du Toit *et al*, 1994); attenuate body weight-loss and tumour growth in MAC16 tumour-bearing mice (Beck *et al*, 1991); up-regulate the expression of E-cadherin (a cell to cell adhesion molecule which suppresses metastasis) (Jiang *et al*, 1995); and to inhibit the *in vitro* growth of three human pancreatic cancer cell lines, MIA, PANC-1 and PaCa-2 (Falconer *et al*, 1994b).

However, the ability of GLA to prevent the progression of cachexia was less pronounced than the effects of EPA (Beck *et al*, 1991). Furthermore, administration of a 10 day continuous infusion of 10g/day GLA, as its lithium salt, followed by oral GLA at a dosage of 3-6g/day, to a group of 18 weight-losing pancreatic cancer patients revealed a down-regulation of the production of TNF- α by PBMC isolated from these patients (Falconer *et al*, 1994b). However, whilst quality of life improved during GLA treatment, only 12% of patients demonstrated unchanged or increased body weight (Fearon *et al*, 1994).

Therefore, whilst administration of GLA may have beneficial effects in cancer cachexia it would seem unlikely that GLA alone would be sufficient to completely inhibit, or reverse, the cachectic process. Thus the utilisation of GLA in combination with other PUFAs or a more conventional form of nutritional support may exhibit greater anti-cachectic properties than GLA alone.

1.2.4.8.2 Eicosapentaenoic Acid (EPA)

Eicosapentaenoic acid, the principal component of fish oil, is a 20:5 n-3 EFA which has been demonstrated to exert a multitude of modulatory actions *in vivo*. The incorporation of n-3 PUFAs, from fish oil feeding, into adipocyte membrane phospholipids has been demonstrated to improve both adipocyte insulin action and glucose metabolism in insulin-resistant rats (Luo *et al*, 1996). Thus administration of fish oil to cachectic individuals who have been found to be glucose intolerant may improve the rate of clearance of exogenous glucose from the blood, and hence correct some of the abnormalities of carbohydrate metabolism frequently noted in cachexia.

Oral administration of fish oil retarded the development of a lupus-like autoimmune disease in MRL-*lpr* mice via an inhibition of synthesis of COX metabolites such as PGE₂ (Kelley *et al*, 1985). Decreased protein kinase C activity in macrophages (Erickson *et al*, 1995), inhibition of the *in vivo* graft versus host response (Jeffery *et al*, 1996), and suppression of the synthesis of IL-1 and TNF- α by mononuclear cells (Caughey *et al*, 1996; Endres, 1996; Meydani, 1996) and IL-6 by PBMCs (Wigmore *et al*, 1997c) have all been observed to occur upon dietary supplementation with marine n-3 fatty acids. Furthermore, n-3 PUFAs have been reported to significantly attenuate the *in vivo* and *in vitro* proliferation of several non-neoplastic (Pell *et al*, 1994; Terano *et al*, 1996;) and neoplastic (Karmali *et al*, 1984; Gabor and Abraham, 1986; Kort *et al*, 1987; Sakaguchi *et al*, 1990; de Bravo *et al*, 1991; Appel and Woutersen, 1994; Falconer *et al*, 1994a; Noguchi *et al*, 1995; Cave Jr, 1996) cell lines, and to inhibit the actions of urokinase, a protease implicated in the invasion and metastasis of malignant cells (du Toit *et al*, 1994).

The progression of cachexia was attenuated in mice and rats bearing experimental tumour models (Tisdale and Dhesi, 1990; Dagnelie *et al*, 1994; Ohira *et al*, 1996), pancreatic cancer patients (Wigmore *et al*, 1996a) and AIDS patients (Hellerstein *et al*, 1996) receiving n-3 PUFA dietary supplementation. This attenuation was accompanied by stabilisation of both skeletal muscle mass and adipose tissue deposits in advanced cancer patients (Wigmore *et al*, 1996b), and a down-regulation of the ubiquitination of muscle proteins, and hence proteolysis, in LLC-IL-6-bearing mice (Ohira *et al*, 1996). Further studies confirmed that administration of high purity EPA alone was sufficient to prevent weight loss in MAC16 tumour-bearing mice (Tisdale and Beck, 1991) LLC-IL-6-bearing mice (Ohira *et al*, 1996) and advanced pancreatic cancer patients (Wigmore *et al*, 1996b).

Thus it would appear that EPA represents the biologically active component of fish oil, and is capable of attenuating host wasting due to either malignancy or AIDS. The exact mechanism of action of EPA is unclear, though the ability to modulate host cytokine secretion and cell responsiveness is probably central to its actions. The prevention of MAC16-induced cachexia by EPA has been attributed to an inhibition of the lipolysis and proteolysis induced by tumour-derived catabolic factors (Beck *et al*, 1991; Tisdale and Beck, 1991), whilst a down-regulation of APPR, and hence reduced REE, may account for the efficacy of EPA in weight-losing cancer patients (Wigmore *et al*, 1997c). Further investigations are required in order to ascertain fully the immuno-modulatory and anti-catabolic mechanisms of action of EPA, and to determine the optimum dose and dosage form. Suggestions have been made that administration of EPA in the form of the free acid may provide the most rapid therapeutic benefits (Hudson and Tisdale, 1994), however, patient tolerance of such a therapy must first be ensured, and if necessary other EPA dosage forms developed.

Viewed objectively, cachexia is a severely debilitating condition, which can, in itself, be self perpetuating and whose cause is still a subject of debate. Any therapy capable of successfully preventing the progression of host wasting will: improve the response of the

patient to more aggressive anti-neoplastic therapies; reduce the propensity of the patient to developing infections; and improve quality of life. At the present time despite the availability of a multitude of pharmacological and nutritional approaches to cachexia no satisfactory treatment has been determined. The most logical therapeutic approaches, such as hyperalimentation, may prove deleterious, further exacerbating tumour growth and disease progression, whilst other strategies may induce weight gain, but are either incapable of repleting host muscle mass, or induce unpleasant side effects. Further investigation of the benefits associated with dietary supplementation using n-3 PUFAs may ultimately provide a well tolerated, low toxicity therapy whereby the nutritional status of the patient may be stabilised, thus significantly improving patient prognosis and enabling more effective treatment of the tumour itself.

1.3 Aims of the Investigation

The principal aim of this investigation is to further elucidate the mechanism(s) involved in the inhibition of MAC16 tumour-derived LMF-induced lipolysis by EPA. Stimulation of lipolysis in isolated murine white adipocytes, and AC activity in adipocyte plasma membrane fractions will be used to determine the optimal inhibitory concentration of EPA and to compare the effects of DHA and AA. Pertussis toxin will be employed to ascertain the involvement of inhibitory G proteins, whilst forskolin will be used to examine the effects of fatty acids upon AC itself. The effects of oral administration of EPA upon tumour factor production will be determined, and the *in vivo* and *in vitro* incorporation of fatty acids into plasma phospholipids, adipocyte membranes and body tissues analysed. Attempts will be made to compare the effects of human- and murine-derived LMF in human and murine white adipocytes, and to further identify the receptor involved in the induction of lipolysis by this factor. Further knowledge of the mechanisms of action of EPA and LMF should enable greater understanding of the role of LMF in the induction of host wasting, and the inhibition of this effect by PUFAs.

Chapter 2 Materials and Buffers

2.1 Animals

Pure strain NMRI (20-25g) and MF1 (40-50g) mice were obtained from our own inbred colonies. Pure strain male BKW mice (40-50g) were purchased from Banting and Kingman, Hull, UK. All animals were fed a rat and mouse breeding diet from Special Diet Services, Witham, Essex and water *ad libitum*. Fragments of the murine adenocarcinoma (MAC16) were implanted into the flanks of male and female NMRI mice by means of a trocar. Tumours were removed once palpable, approximately 14 days post transplantation, and before weight loss exceeded 25% of total body weight.

2.2 Chemicals

Amersham International Ltd, Buckinghamshire, UK.

[8-³H] Adenosine 3',5' - cyclic phosphate (cAMP), ammonium salt

(specific activity 0.37-1.1TBq/mmol)

[α -³²P] Adenosine 5' triphosphate (ATP) (specific activity approx 1.11TBq/mmol)

N,N'-Bis-methylene acrylamide

Bio-Rad Laboratories, Richmond, CA.

Acrylamide

Ammonium persulphate

Fisher Scientific Apparatus, Leicestershire, UK.

Chloroform

Ethanol

Glacial acetic acid

Hydrochloric acid

Methanol

Optiphase HiSafe 3

Perchloric acid

Oxoid, Basingstoke, Hampshire, UK.

Phosphate buffered saline tablets

Santa Cruz Biotechnology Inc.

Anti G α _{i-3}

Anti G α _{s/olf}

Sigma-Aldrich Co Ltd, Dorset, UK.

Adenosine 3':5'-cyclic monophosphate

Adenosine 5'-triphosphate, trisodium salt

Anti-rabbit IgG

Arachidonic acid

Authentic fatty acid methyl ester standards

14% Boron trifluoride in methanol

Bovine serum albumin

Bromophenol Blue

Butylated hydroxytoluene

Calcium chloride

Collagenase, Type II

Coomassie Brilliant Blue R250 stain

Creatine phosphate

Creatine phosphokinase

Dithiothreitol

Docosahexaenoic acid

Dowex 50X8-400 resin

Ethylene glycol-bis(β aminoethylether) N,N,N',N' tetracetic acid
Eicosapentaenoic acid
Forskolin
Glucose
Glycerokinase
Glycerol
Glycine
n-Hexane
Imidazole
3-isobutyl-1-methyl xanthine
Isoprenaline
Kodak GBX developer and replenisher
Kodak GBX fixer and replenisher
Lactate dehydrogenase
Magnesium chloride
Magnesium sulphate
Margaric Acid
 β -mercaptoethanol
 α -Nicotinamide adenine dinucleotide - reduced form (NADH)
 β -Nicotinamide adenine dinucleotide (NAD)
Neutral alumina
Percoll
Pertussis toxin
Phenylmethylsulphonyl fluoride
Phosphoenolpyruvate
Potassium chloride
Potassium dihydrogen orthophosphate
Propranolol
Pyruvate kinase

Sodium bicarbonate
Sodium chloride
Sodium dodecyl sulphate
Sodium hydroxide
Sucrose
N,N,N',N'-Tetramethylethylenediamine
Triethanolamine
Trizma base

2.3 Buffers

2.3.1 Krebs Ringer Bicarbonate Solution

Sodium chloride	118mM
Potassium chloride	5mM
Calcium chloride	2mM
Potassium dihydrogen orthophosphate	1mM
Magnesium sulphate	1mM
Sodium bicarbonate	25mM

2.3.2 Glycerol Assay Buffer

Triethanolamine	100mM
Magnesium sulphate	2mM
Phosphoenolpyruvate	0.4mM
α -Nicotinamide adenine dinucleotide (NADH)	0.25mM
ATP	1.2mM
Pyruvate kinase	1.0units/ml
Lactate dehydrogenase	7.0units/ml

The constituents were dissolved in deionised water and the pH adjusted to 7.4 using concentrated hydrochloric acid. The reaction was initiated by the addition of 1 unit of glycerokinase and allowed to proceed for 15min.

2.3.3 *Q Sepharose Buffer 1*

Tris-HCl, pH 8.0	10mM
DTT	1mM
EGTA	0.5mM
PMSF	0.5mM

2.3.4 *Q Sepharose Buffer 2*

Tris-HCl, pH 8.0	10mM
DTT	1mM
EGTA	0.5mM
PMSF	0.5mM
Sodium chloride	0.2mM

2.3.5 *Phosphate Buffer, pH 8.0*

1M Disodium hydrogen phosphate	93.2ml
1M Sodium dihydrogen phosphate	6.8ml

2.3.6 *Superdex Buffer*

Phosphate buffer, pH 8.0	50mM
Sodium chloride	0.3mM
DTT	1mM
EGTA	0.5mM
PMSF	0.5mM

2.3.7 Sucrose Buffer, pH 7.4

Sucrose	0.25M
EGTA	2mM
Tris-HCl	10mM

2.3.8 Concentrated Sucrose Solution, pH 7.4

Sucrose	2M
EGTA	8mM
Tris-HCl	80mM

2.3.9 Sodium Chloride Buffer, pH 7.4

NaCl	0.15M
EGTA	1mM
Tris-HCl	10mM

2.3.10 Plasma Membrane Buffer, pH 7.4

Sucrose	0.25M
EGTA	2mM
Tris-HCl	10mM
PMSF	4 μ M

2.3.11 44% Acrylamide/Bis Stock Solution

Acrylamide	44g
N,N'-Bis-methylene acrylamide	0.8g
Distilled water	to 100ml

2.3.12 15% Resolving Gel

44% Acrylamide/Bis Stock Solution	3.7ml
1.5M Tris-HCl, pH 8.8	3.75ml
Distilled water	3.95ml
10% SDS	0.3ml
TEMED	30 μ l
10% APS	40 μ l

2.3.13 30% Acrylamide/Bis Stock Solution

Acrylamide	30g
N,N'-Bis-methylene Acrylamide	0.8g
Distilled water	to 100ml

2.3.14 5% Stacking Gel

30% Acrylamide/Bis Stock solution	1.0ml
1M Tris-HCl, pH 6.8	1.5ml
Distilled water	3.2ml
10% SDS	60 μ l
TEMED	16 μ l
10% APS	20 μ l

2.3.15 Running Buffer (10x), pH 8.3-8.5

Trizma base	30.29g
Glycine	144.3g
10% SDS	100ml
Distilled water	to 1 litre

Dilute 100ml of running buffer with 900ml distilled water prior to use.

2.3.16 Denaturing Sample Buffer (2x)

Tris-HCl, pH 6.8	0.0625M
Glycerol	10%
SDS 1%	
Bromophenol blue	0.01%
β -mercaptoethanol	5%

Dilute 1:1 with sample.

2.3.17 Blotting Buffer (10x)

Trizma base	30.3g
Glycine	144g
SDS	15g
Distilled water	to 1 litre

For use :-	10x Blotting buffer	100ml
	Methanol	200ml
	Distilled water	to 1 litre

2.3.18 Coomassie Blue Gel Stain

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

2.3.19 Coomassie Blue Gel Destain

Acetic acid	10%
Methanol	25%

Chapter 3 Methods

3.1 *Incorporation of Eicosapentaenoic Acid into Plasma Phospholipids and Body Organs*

3.1.1 *Oral Administration of Eicosapentaenoic Acid*

3.1.1.1 *Administration of Eicosapentaenoic Acid to Pancreatic Cancer Patients*

Patients with unresectable adenocarcinoma of the pancreas received oral dietary supplementation with 1g soft gelatine capsules of fish oil (MaxEPA[®], Seven Seas Health Care, Hull, UK) containing 18% w/w EPA and 12% w/w DHA. The initial dose was 2g/day, increasing by 2g/week to a maximum dose of 16g/day (Wigmore *et al*, 1996a). Plasma samples were obtained from patients before supplementation and 1 month after commencement of the trial and stored at -20°C. Subsequent samples were obtained from patients receiving 95% pure EPA (0.5g gelatine capsules) initial dose 91g over 4 weeks followed by a maintenance dose of 6g/day (Wigmore *et al*, 1996b) and a fish oil containing food supplement (Ross Products Ltd, Columbus, Ohio, USA) consisting of 16.1g protein, 49.7g carbohydrate, 6.5g fat/can at a dose of 2 cans/day, equivalent to 2g EPA, 1g DHA, 620kcal/day.

3.1.1.2 *Administration of Eicosapentaenoic Acid to NMRI Mice*

Non tumour-bearing male NMRI mice, approximately 20g, were post orally dosed for 24h with 1.25g/kg EPA 90 (Callanish, UK). Blood was extracted from mice by cardiac puncture under anaesthesia using Boyle's apparatus and a halothane, oxygen and nitrous oxide mixture. Mice were subsequently killed by cervical dislocation, their epididymal fat pads and gastrocnemius muscles were removed and all samples stored at -20°C.

3.1.2 *Extraction of Fatty Acids*

Fatty acids were extracted from body organs and plasma phospholipids according to the method of Folch *et al* (1957). Briefly, material was homogenised in 10 volumes of 2:1 vol/vol chloroform:methanol to which 0.01 volumes of 2% butylated hydroxytoluene in ethanol (an antioxidant) and 50 μ g of margaric acid, as an internal standard, were added. The mixture was separated into organic and non-organic phases by centrifugation after mixing with 1ml methanol and 0.2 volumes distilled water. The organic phase, which contained the lipid extract, was then aspirated and saponified by the addition of 5% sodium hydroxide in 50% methanol and heating to 100°C for 45-60min in an atmosphere of nitrogen. After cooling, the fatty acids were methylated by heating at 80°C for 5min in the presence of 14% boron trifluoride in methanol, extracted twice using hexane:chloroform (4:1 vol/vol), evaporated *in vacuo*, redissolved in 200 μ l n-hexane and stored under nitrogen at -70°C until analysed.

3.1.3 *Analysis of Fatty Acids*

The fatty acid methyl esters were analysed using a Hewlett Packard 5890 Series II gas chromatograph fitted with a narrowbore (0.25mm) DB32 column (J & W Scientific, Fisons, UK) and a flame ionisation detector at 240°C. The temperature program increased at a rate of 5°C/min from 180°C-220°C which was then maintained for 15min. The column was run with helium as the carrier gas at a linear velocity of 38.5cm/second with a split of 1:100. Fatty acid peaks were identified by comparison of the retention times with those of authentic standards. Relative percentage fatty acids present were calculated for patient plasma samples, murine serum samples and murine body organs.

3.2 Effect of Eicosapentaenoic Acid on the Production of Lipid Mobilising Factor by the MAC16 Tumour

3.2.1 Oral Administration of Eicosapentaenoic Acid to MAC16 Tumour-Bearing Mice

Male MAC16 tumour-bearing NMRI mice, with established body weight loss, were dosed post-orally with 1.25g/kg EPA 90 (Callanish, UK) for 24h. Mice were killed by cervical dislocation, their tumours dissected out and stored at -20°C until required.

3.2.2 Determination of Protein Concentration

Protein was determined by the method of Bradford (1976), using the Bio-Rad Protein reagent, a dye-binding assay based on the differential colour change of Coomassie Brilliant Blue G-250 in response to various concentrations of protein. Briefly, 10µl of sample was added to a solution of distilled water plus 200µl Bio-Rad reagent to give a total volume of 1ml. These were mixed and the absorbance of the mixture measured at 595nm in a Beckman DU-70 Spectrophotometer against a blank containing distilled water and Bio-Rad reagent only.

$$\frac{\text{Sample absorbance} - \text{Control absorbance}}{0.053} \times \frac{1}{10} = \mu\text{g of protein}/\mu\text{l}$$

3.2.3 Purification of Murine Lipid Mobilising Factor

A proteinaceous extract was isolated from the MAC16 tumour on the basis of its ability to induce lipolysis in murine white adipocytes *in vitro* using the method of McDevitt *et al*, (1995). Tumours from both EPA dosed and undosed mice were utilised in order to study the effects of EPA on the production of lipid mobilising factor by MAC16.

3.2.3.1 Homogenisation of Tumours

The dissected MAC16 tumours were homogenised in 5ml/g tumour Q Sepharose buffer 1 (QS1) (Section 2.3.3). The resulting homogenate was centrifuged for 10min at 4000rpm

in an Heraeus Sepatech Megafuge 1.0 to remove debris, and the protein concentration determined (Section 3.2.2).

3.2.3.2 Batch Extraction

The anion exchange resin DEAE cellulose (5g/g tumour) was equilibrated using 100mM Tris-HCl, pH 8.0, and further washed three times with 10mM Tris-HCl, pH 8.0, allowing the resin to sediment before pouring off the supernatant. The tumour homogenate was diluted 2:1 with QS1, added to the equilibrated DEAE cellulose and stirred at 4°C for 30min. The resulting slurry was centrifuged at 4000rpm for 10min, the supernatant discarded and the cellulose pellet resuspended in 0.3M sodium chloride. The slurry was again centrifuged and the supernatant retained. The pellet was resuspended a second time in 0.3M sodium chloride, and stirred for 30min at 4°C prior to centrifugation.

3.2.3.3 Amicon Ultrafiltration

The pooled salt washes from Section 3.2.3.2 were concentrated to a volume of approximately 50ml in an Amicon Ultrafiltration unit (Amicon Ltd, UK) employing a filtration membrane with a molecular weight cut off of 10,000. The concentrated extract was diluted 1:3 with deionised water containing 1mM DTT, 0.5mM EDTA and 0.5mM PMSF, and the volume reduced again. This was repeated three times to ensure total removal of sodium chloride. The final 30-50ml concentrate was freeze dried and stored at -20°C.

3.2.3.4 Q Sepharose Anion Exchange Chromatography

The freeze dried residue from Section 3.2.3.3 was resuspended in a small volume of QS1 buffer (Section 2.3.3), centrifuged at 13,000rpm for 10min and its protein concentration determined (Section 3.2.2). The sample was loaded onto a column packed with the anion exchange resin Q Sepharose, which had been previously equilibrated with QS1 buffer, and allowed to bind to the matrix. The column was washed with one column volume of QS1 and the eluate collected to verify the absence of lipid mobilising activity. Proteins bound to

the column were fractionated using a sodium chloride gradient (0-0.2M) created with Q Sepharose 2 (QS2) buffer (Section 2.3.4). The elution rate was 0.5ml/min and 3ml fractions were collected by an automated fraction collector. Residual proteins were removed by extensive washing of the column with 3M sodium chloride. A 100 μ l aliquot of each fraction was assayed for lipolytic activity (Section 3.2.4) and active fractions pooled and freeze dried.

3.2.3.5 Superdex Gel Exclusion Chromatography

The freeze dried fractions from Section 3.2.3.4 were redissolved in 1.2ml Superdex buffer (Section 2.3.6), centrifuged at 13,000rpm for 10min and the protein concentration measured (Section 3.2.2). A 1ml sample was then injected onto a prepacked Superdex column connected to a fast protein liquid chromatography (FPLC) system running at 1ml/min and sixty 2ml fractions were collected. The void volume was discarded and 100 μ l aliquots of each fraction assayed for lipolytic activity (Section 3.2.4). Active fractions were pooled and re-equilibrated in QS1 using Amicon ultrafiltration (Section 3.2.3.3).

3.2.3.6 Comparison of Lipid Mobilising Activity Purified from MAC16 Tumours of EPA Dosed and Undosed Mice

The protein concentration of the purified lipid mobilising factor was determined (Section 3.2.2) and isolated murine adipocytes incubated for 2h with known concentrations of factor purified from the tumours of both dosed and undosed male NMRI mice (Section 3.2.4). At the end of the incubation period glycerol release was measured (Section 3.2.4.3) and the effects of the two factors compared.

3.2.4 Determination of Lipid Mobilising Activity

3.2.4.1 Isolation of Murine Epididymal Adipocytes

Male BKW, or NMRI, mice were killed by cervical dislocation and their epididymal fat pads removed. Each pair of fat pads was roughly chopped, gassed with 95% O₂/5% CO₂ and incubated, with shaking, for 30min at 37°C in a 1ml aliquot of Krebs Ringer

Bicarbonate buffer (Section 2.3.1) containing 3% bovine serum albumin and 4mg/ml collagenase, to produce a homogeneous single cell suspension.

3.2.4.2 Lipolytic Assay

The isolated adipocytes from 3.2.4.1 were then separated from other non-adipocyte material and collagenase by washing three times in non-supplemented Krebs buffer, each time allowing viable cells to float to the surface. Following the final wash, murine adipocytes were suspended in Krebs + 3% BSA at a concentration of $1-2 \times 10^5$ cells/ml, cell number being enumerated using a Neubauer haemocytometer. Samples of the cell suspension (1ml) were removed, added to microfuge tubes containing the required concentration of either isoprenaline or lipolytic factor, gassed with 95% O₂/5% CO₂ and incubated for 2h at 37°C with shaking. Control samples containing adipocytes alone were also analysed in order to determine any endogenous lipolysis.

At the end of the incubation period 0.5ml of the incubation medium was added to 0.5ml 10% w/v perchloric acid and the mixture shaken to ensure deproteinisation. The precipitated protein was removed by centrifugation at 13,000rpm for 1min and neutralised using 40% w/v potassium hydroxide.

3.2.4.3 Glycerol Determination

The release of glycerol from the adipocytes during the incubation period was used as a measure of lipolysis. The concentration of glycerol present in the neutralised supernatant was determined by the method of Weiland (1974). Briefly, a 200 μ l aliquot of supernatant was added to 830 μ l of Glycerol Assay buffer (Section 2.3.2) and the change in absorbance produced by the conversion of NADH to NAD⁺ (Figure 3.1) measured over 15min following the addition of 1 unit of glycerokinase to the sample. Samples were measured against a control of glycerol buffer only.

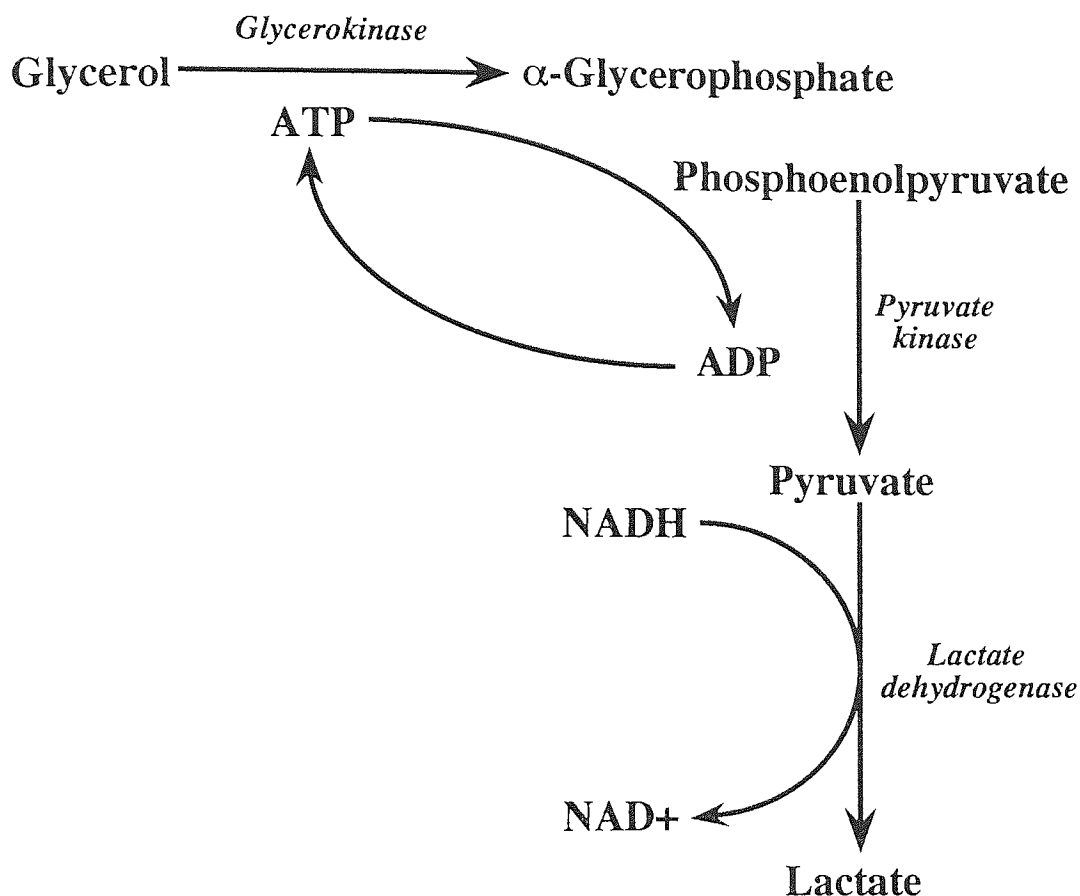


Figure 3.1 Glycerol reaction

Calculation from Beer-Lamberts Law :

$$\frac{\text{Change in sample absorbance} - \text{Change in control absorbance}}{\text{Extinction coefficient (6.22) of NADH}} \times \frac{\text{Dilution factor (10)}}{\mu\text{mole}/10^5 \text{ adipocytes/ml/2h}} = [\text{Glycerol}]$$

3.3 Comparison of the Effects of Isoprenaline, Murine and Human Lipid-Mobilising Activity in Murine and Human White Adipocytes.

A proteinaceous factor, identical to that purified from the MAC16 tumour, was purified from the urine of cachectic cancer patients according to the method of Cariuk *et al* (1997). The effects of this factor together with the effects of isoprenaline and the MAC16 derived LMF were compared in both murine and human white adipocytes.

3.3.1 Purification of Human Lipid-Mobilising Factor

Urine collected from cachectic cancer patients was centrifuged at 13000rpm for 30min to remove all particulates and reduced in volume (Amicon ultrafiltration, Section 3.2.3.3). Superfluous proteins were precipitated by the slow addition of 38% w/v ammonium sulphate at 4°C with stirring. After overnight equilibration, the precipitated proteins were removed by centrifugation at 5000rpm for 20min. The resulting supernatant was desalted as described in Section 3.2.3.3 and further purified using Q Sepharose anion exchange chromatography (Section 3.2.3.4). Active fractions were determined by lipolytic assay (Section 3.2.4.2), pooled and re-equilibrated in QS1 using Amicon ultrafiltration (Section 3.2.3.3).

3.3.2 Isolation of Human Omental Adipocytes

Human omental adipose tissue was removed under general anaesthesia and transported immediately to the laboratory. Fragments of tissue (roughly equivalent in size to a pair of murine epididymal fat pads) were digested to produce a single cell suspension of adipocytes by incubation at 37°C for 30min in a shaking water bath in a 1ml aliquot of Krebs Ringer Bicarbonate Buffer (Section 2.3.1) supplemented with 4% bovine serum albumin, 1g/l glucose and 1.5mg/ml collagenase.

3.3.3 Comparison of the Effects of Isoprenaline, Murine and Human Lipid-Mobilising Activity in Murine and Human White Adipocytes.

The protein concentrations of the purified murine and human lipid-mobilising factors were determined (Section 3.2.2). Isolated murine and human adipocytes (Sections 3.2.4.1 and 3.3.2) were suspended in Krebs Ringer Bicarbonate buffer (Section 2.3.1) containing either 3% BSA or 4% BSA + 1g/l glucose, respectively at a concentration of $1-2 \times 10^5$ cells/ml and incubated for 2h with known concentrations of either isoprenaline, murine LMF or human LMF (Section 3.2.4.2). At the end of the incubation period glycerol release was measured (Section 3.2.4.3) and the effects of the three agonists compared.

3.4 Effects of Fatty Acids or β -adrenergic Antagonists on Lipid-Mobilising Factor Induced Lipolysis

3.4.1 Effect of Polyunsaturated Fatty Acids on Lipid-Mobilising Factor Stimulated Lipolysis in Murine White Adipocytes

A single cell suspension of murine white adipocytes was prepared as described in Section 3.2.4.1. Adipocytes were incubated for 15min at 37°C with either EPA, DHA or AA (dissolved in ethanol) prior to the addition of isoprenaline or murine lipid-mobilising factor, purified as described in Section 3.2.3.1. Glycerol release was subsequently determined (Section 3.2.1.4).

3.4.2 Effect of the β_3 -adrenergic Antagonist SR 59230A on Isoprenaline and Factor Stimulated Lipolysis in Murine White Adipocytes

Adipocytes isolated as described in Section 3.2.4.1 were washed 3 times in Krebs Ringer Bicarbonate buffer (Section 2.3.1) containing the required concentration of SR 59230A. After each wash the fat cells were allowed 5min to float to the surface before being harvested for the next wash. Following the final wash the adipocytes were diluted using Krebs + 3% BSA to a concentration of approximately $1-2 \times 10^5$ cells/ml and dispensed into microfuge tubes. The required concentration of SR 59230A was then added to each sample, and the tubes incubated for 15min at 37°C prior to the addition of isoprenaline or murine lipid-mobilising factor (Section 3.2.4.2).

3.5 Effects of Fatty Acids or β -adrenergic Antagonists on Isoprenaline or Lipid Mobilising Factor Stimulated Adenylate Cyclase Activity in Isolated Adipocyte Plasma Membranes

3.5.1 Isolation of Murine Adipocyte Plasma Membranes

Adipocyte plasma membranes were isolated according to the method of Belsham *et al* (1980). The isolated murine adipocytes from Section 3.2.4.1 were washed three times

with Sucrose Buffer (Section 2.3.7) and suspended in approximately 20ml of this buffer. The cells were disrupted by rapid aspiration through a Swinny filter. The sheared cell suspension was centrifuged for 5min at 1000rpm in an Heraeus Sepatech Megafuge 1.0. The resulting fat cake was carefully removed, the pellet resuspended in the infranant and centrifuged at 30,000g at 4°C for 1h in an AP Pegasus 65 Centrifuge. The supernatant was discarded and the pellet resuspended in 400 μ l Sucrose Buffer. The resuspended pellet was then added to a mixture of percoll, concentrated sucrose solution (Section 2.3.8) and sucrose buffer, in the proportions 7:1:32 respectively, to a total volume of 8ml, and centrifuged at 10,000g for 30min at 4°C.

The top 1ml of the percoll gradient, which contained the plasma membrane fraction, was washed three times with Sodium Chloride Buffer (Section 2.3.9) centrifuging at 10,000g for 2min at 4°C for each wash. The washed plasma membranes were then resuspended in 200-400 μ l Plasma Membrane Buffer (Section 2.3.10), the protein concentration determined (Section 3.2.2) and adjusted to 1-2mg/ml before the membranes were snap frozen in liquid nitrogen. Frozen membranes were stored either overnight in liquid nitrogen, or at -70°C for longer periods.

3.5.2 *Determination of Adenylate Cyclase Activity*

3.5.2.1 *Adenylate Cyclase Assay*

Adenylate cyclase activity in isolated adipocyte plasma membranes was determined using the method of Salomon *et al* (1974). The incubation mixture contained 25mM Tris-HCl, pH 7.4, 5mM MgCl₂, 8mM creatine phosphate, 16U/ml creatine phosphokinase, 1mM cAMP, 1mM 3-isobutyl-1-methyl xanthine, 1mM [α -³²P] ATP (3 μ Ci, specific activity 30 μ Ci/mmol) and 10 μ M GTP. The reaction was initiated by the addition of cold plasma membranes to the assay mixture, to give a total volume of 100 μ l which was incubated at 30°C for 10min. The reaction was terminated by the addition of 100 μ l of a stopping

solution containing 40mM ATP, 1.4mM cAMP and 2% SDS. In order to calculate the recovery of cAMP after its isolation [³H] cAMP (1μCi, 50μl) was also added.

3.5.2.2 Isolation of Cyclic AMP

Cyclic AMP produced by the action of AC on ATP was isolated using Method C of Salomon *et al* (1974). Essentially, 0.75ml distilled water was added to each reaction tube from 3.4.2.1, the contents of each tube were then pipetted into columns containing 1ml Dowex 50WX8-400 resin and the eluate discarded. The eluates from two successive 1ml distilled water washes were also discarded. Distilled water (3ml) was then added to each column and the eluate collected into polypropylene tubes containing 200μl 1.5M imidazole-HCl, pH 7.2, to give a pH of approximately 7.5 on dilution.

The contents of these tubes were then decanted into columns containing 0.6g neutral alumina which had been previously washed with 8ml 0.1M imidazole-HCl, pH 7.5. The eluate was collected directly into scintillation vials containing 12ml Optiphase HiSafe 3. Once the columns had completely drained an additional 1ml 0.1M imidazole-HCl, pH 7.5 was added and collected into the scintillation vials.

The Dowex columns were cleaned by the addition of 3ml 1M hydrochloric acid after each experiment, and washed with 10ml distilled water on the next day of use. Alumina columns were recycled by washing with 8ml 0.1M imidazole-HCl between experiments. A dual count was performed on each sample using a Packard Tricarb 2000CA Liquid Scintillation Analyser and the production of [³²P] cAMP from [α -³²P] ATP calculated as follows, background was determined by counting 4ml imidazole-HCl in 12ml scintillation fluid.

1. Subtract background counts per min (cpm) for [³H] and [³²P] from all cpm values obtained.

2. Calculate Specific Activity (SA) of [α - ^{32}P] ATP in the assay:

$$\text{SA} = \frac{[^{32}\text{P}] \text{ ATP (100\% dilution) cpm}}{\text{Assay [ATP]} \mu\text{M}}$$

3. Calculate the Assay Constant (AC):

$$\text{AC (cpm/pmole/mg)} = \text{SA} \times [\text{membrane protein}] \text{ mg}$$

4. Calculate the spillover of [^{32}P] into the [^3H] channel using the [^{32}P] standard:

$$\text{Spill Over (SO)} = \frac{\text{ATP standard } [^3\text{H}] \text{ cpm}}{\text{ATP standard } [^{32}\text{P}] \text{ cpm}}$$

$$[^{32}\text{P}] \text{ cAMP pmole/mg} = \frac{\text{Sample } [^{32}\text{P}] \text{ cpm} \times \text{cAMP standard } [^3\text{H}] \text{ cpm}}{\text{AC} \times (\text{sample } [^3\text{H}] \text{ cpm} - (\text{sample } [^{32}\text{P}] \text{ cpm} \times \text{SO}))}$$

$$\text{Adenylate Cyclase Activity} = \frac{[^{32}\text{P}] \text{ cAMP}}{\text{(pmoles/mg membrane/min)} \quad \text{assay time (min)}}$$

3.5.3 Effect of Polyunsaturated Fatty Acids on Factor Stimulated Adenylate Cyclase Activity in Murine White Adipocyte Plasma Membranes

Isolated adipocyte plasma membranes (Section 3.5.1) were incubated for 30min at room temperature with either EPA, DHA or AA (dissolved in ethanol). At the end of the pre-incubation period, membranes were added to the assay mix and AC activity determined as described in Section 3.5.2.

3.5.4 Effect of the β_3 -adrenergic Antagonist SR 59230A on Isoprenaline and Factor Stimulated Adenylate Cyclase Activity in Murine Adipocyte Plasma Membranes

Isolated adipocyte plasma membranes (1-2mg/ml) were pre-incubated for 15min at 30°C with SR 59230A dissolved in distilled water. Membranes were then added to the incubation mixture (Section 3.5.2) and AC activity stimulated using either isoprenaline or lipolytic factor.

3.5.5 Effect of Oral Administration of Eicosapentaenoic Acid on Isoprenaline, Factor and Forskolin Stimulated Adenylate Cyclase Activity in Murine White Adipocyte Plasma Membranes

Male NMRI mice were dosed post-orally with 1.25g/kg EPA 90 (Callanish, UK) for 24h. Mice were killed by cervical dislocation, epididymal fat pads removed, adipocyte plasma membranes prepared (Section 3.5.1) and AC activity determined (Section 3.5.2).

3.5.6 Effect of Oral Administration of Eicosapentaenoic Acid on Isoprenaline and Factor Stimulated Adenylate Cyclase Activity in Murine White Adipocyte Plasma Membranes Isolated from Cachectic Mice

Male NMRI mice bearing the MAC16 tumour, and with established weight loss, were dosed post-orally with 1.25g/kg EPA 90 (Callanish, UK) for 24h. Mice were killed by cervical dislocation, epididymal fat pads removed and adipocyte plasma membranes prepared (Section 3.5.1). Adipocyte membranes were also prepared from non tumour-bearing mice, and from undosed MAC16-bearing mice. Adenylate cyclase activity in response to both isoprenaline and lipid mobilising factor was determined (Section 3.5.2).

3.6 Effect of Pertussis Toxin on the Inhibition of Lipolysis and Adenylate Cyclase Activity by Eicosapentaenoic Acid

3.6.1 Pre-activation of Pertussis Toxin

Pertussis toxin was pre-activated by incubation at 37°C for 10min in a freshly prepared solution containing 200mM sodium phosphate, pH 7.4, 0.5mM DTT and 100 μ M ATP at a concentration of 250 μ g/ml (Rothenburg *et al*, 1988).

3.6.2 Effect of Pertussis Toxin on the Inhibition of Lipolysis by Eicosapentaenoic Acid

Isolated murine white adipocytes were washed with Krebs Ringer Bicarbonate buffer and 1ml aliquots dispensed into microfuge tubes. Pertussis toxin was activated as described in Section 3.6.1 and added to half of the tubes at a concentration of 500ng/ml. The tubes

were gassed with 95% O₂/5% CO₂ and incubated with pertussis toxin for 1h at 37°C. The adipocytes were then treated with EPA (331µM) for 15min at 37°C prior to the addition of either isoprenaline or lipid mobilising factor and incubation of the cells for 90min at 37°C. Glycerol release was subsequently determined as detailed in Section 3.2.4.4.

3.6.3 Effect of Pertussis Toxin on the Inhibition of Adenylate Cyclase Activity by Eicosapentaenoic Acid

Pertussis toxin was pre-activated as described in section 3.6.1. Isolated adipocyte plasma membranes (Section 3.5.1) were pre-incubated with 25µg/ml pertussis toxin, 5mM MgCl₂, 1µM ATP and 5µM NAD for 30min at 30°C. Membranes were then treated with 100µM EPA (dissolved in ethanol) before being added to the AC assay mixture (Section 3.5.2).

3.7 Western Blotting

3.7.1 Preparation of Protein Samples

3.7.1.1 Preparation of MaxEPA Trial Patient Plasma

The protein concentration was determined (Section 3.2.2) and adjusted to 1mg/ml. The sample (1µg for immunodetection, 10µg for Coomassie staining) was then denatured by heating for 5min at 100°C in an equivalent volume of Sample Buffer (Section 2.3.16), cooled and centrifuged at 13,000rpm for 1min in an Heraeus Sepatech Biofuge 1.3. High Molecular Weight Standards (Sigma-Aldrich Co Ltd, Dorset, UK) were prepared in a similar fashion and loaded onto the gel with the samples in order to permit the determination of the molecular weights of the proteins present in the sample.

3.7.1.2 Immunoprecipitation of Cachectic Factor

In an attempt to reduce the immunoglobulin content of plasma, and thus eradicate non-specific antibody binding, 10µg of plasma protein was mixed with 25µg monoclonal antibody and 50µl Protein A Sepharose. The samples were agitated overnight at 4°C and

centrifuged for 1min at 13,000rpm in an Heraeus Sepatech Biofuge 1.3. The supernatant was then carefully removed, 10 μ l of Sample Buffer (Section 2.3.16) added to the pellet, the sample prepared as described in Section 3.6.1 and Western Blotted.

3.7.1.3 Preparation of Adipocyte Plasma Membrane Samples

Plasma membranes were isolated from non tumour-bearing NMRI mice, from MAC16 tumour-bearing mice who had lost 0-20% of their original body weight and from MAC16 tumour-bearing mice with 10% weight-loss who had received 1.25g/kg EPA for 24h. Membranes (1 μ g) were denatured by heating in Sample buffer (Section 2.3.16), cooled and centrifuged.

3.7.2 SDS-Polyacrylamide Gel Electrophoresis

Samples were electrophoretically analysed according to the method of Laemmli (1970) using a discontinuous buffer system in SDS/Polyacrylamide gels. Gels containing 15% acrylamide were prepared as described in Section 2.3.12 and poured into a pre-made gel plate apparatus (Bio-Rad Laboratories, Richmond, CA) which had been cleaned with 70% ethanol. The gel was overlaid with distilled water and allowed to polymerise (30min). The distilled water was then removed, the 5% stacking gel (Section 2.3.14) poured on top of the running gel, and an appropriate gel comb inserted.

After polymerisation was completed, approximately 30min, the gel plates were placed into the electrophoretic apparatus, the reservoirs filled with Running Buffer (Section 2.3.15) and the protein samples loaded into the wells created in the stacking gel by the gel comb. Samples were electrophoresed at 180V until the protein/dye front reached the base of the running gel.

3.7.3 *Coomassie Brilliant Blue Staining*

For routine visualisation of the proteins present in the samples, gels were placed in a staining solution of Coomassie Brilliant Blue (Section 2.3.18) and stained for 2h at room temperature with gentle agitation. Gels were destained for 2-3h with several changes of Destaining Solution (Section 2.3.19). Gels were stored by drying between cellophane under vacuum at 80°C for 2h.

3.7.4 *Transfer of Electrophoresed Proteins to Nitrocellulose*

After electrophoresis the gel was soaked for 15min in Blotting Buffer (Section 2.3.17) together with a piece of hydrated nitrocellulose membrane. The gel and membrane were then sandwiched together between 3MM paper (Figure 3.2) and the proteins transferred for 2h at 80V with cooling. Non-specific sites on the membrane were blocked by incubating overnight in 5% Marvel in PBS + 0.1% Tween 20 at 4°C.

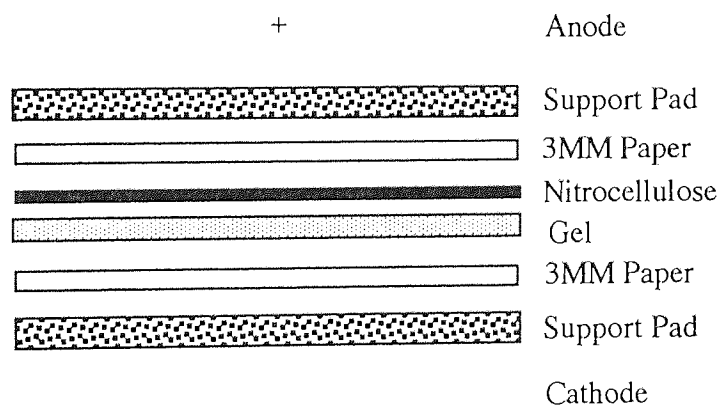


Figure 3.2 Procedure for transfer of electrophoresed proteins to a nitrocellulose membrane

3.7.4.1 Immunodetection of Western Blots

3.7.4.1.1 MaxEPA Trial Patient Plasma Samples

The blocked nitrocellulose membrane was washed for 15min and 2 x 5min in a large volume of PBS + 0.5% Tween 20 at room temperature. Next the membrane was incubated at room temperature for 1h with Biotin tagged monoclonal antibody in PBS + 0.1% Tween 20, at a concentration of 10 μ g/ml. After washing in PBS + 0.1% Tween 20 for 1 x 15min and 2 x 5min the membrane was incubated in a 1:1500 dilution of a pre-formed Horseradish Peroxidase-Streptavidin complex for a further hour. The blot was then washed for 3 x 15min and 2 x 5min prior to visualisation.

3.7.4.1.2 Adipocyte Plasma Membrane Samples

The blocked nitrocellulose membrane was washed for 1 x 15min and 2 x 5min in a large volume of PBS + 0.5% Tween 20 at room temperature. Next the nitrocellulose membrane was incubated at room temperature for 1h with polyclonal anti-G α _i or anti-G α _s in PBS + 0.1% Tween 20, at a concentration of 1 μ g/ml. After washing in PBS + 0.1% Tween 20 for 1 x 15min and 2 x 5min the membrane was incubated in a 1:1500 dilution of Horseradish Peroxidase-conjugated anti-rabbit IgG complex for a further hour. The blot was then washed for 3 x 15min and 2 x 5min prior to visualisation.

3.7.4.2 Visualisation of Western Blots

Blots were visualised using the ECL™ Western Blotting Detection System (Amersham International PLC, Buckinghamshire, UK). This system is based on the oxidation of luminol by horseradish peroxidase to produce light emission which may be detected by short exposure to blue-light sensitive autoradiography film.

The blots were covered in a mixture of equal volumes of reagents 1 and 2 for 1min at room temperature, drained, wrapped in Saran wrap and taped into a film cassette. Hyperfilm ECL™ was then exposed to the membrane for 30 seconds to 20min, depending on the

concentration of target protein present. Blots were developed by placing in Kodak Developer & Replenisher for 5min and fixed by immersion in Kodak Fixer & Replenisher for 5min.

Chapter 4 - Results

4.1 Investigation into the Effect of the Selective β_3 -antagonist SR 59230A upon the Actions of the MAC16-Derived Lipid-Mobilising Factor

4.1.1 Introduction

It has been demonstrated that the MAC16 tumour-derived LMF induces lipolysis in isolated murine white adipocytes via an elevation of intracellular cAMP, the consequence of a stimulation of adenylate cyclase activity (Tisdale and Beck, 1991). It has been postulated that the lipolytic actions of LMF may be mediated through β -adrenoceptors located on the surface of the adipocyte, and that the receptors involved may be atypical, or β_3 -adrenergic receptors (Khan, 1996). The *SS*-enantiomer 3-(2-ethylphenoxy)-1-[(1*S*)-1,2,3,4-tetrahydronaphth-1-ylaminol]-(2*S*)-2-propanol oxalate (SR 59230A) has been proposed to be the first selective β_3 -adrenergic receptor antagonist, displaying an approximately ten fold greater affinity for β_3 -adrenoceptors over β_2 -adrenoceptors, and has been reported to counteract the effects of the β_3 -adrenergic agonists SR 58611A (ethyl-[(7*S*)-7-[[*(2R)*-2-(3-chlorophenyl)-2-hydroxyethyl] amino]-5,6,7,8-tetrahydronaphth-2-yl] oxyacetate hydrochloride and CGP 12177 ((-)-4-(3-*t*-butylamino-2-hydroxypropoxy)benzimidazol-2-one) in rat brown adipocytes without affecting forskolin-induced cAMP accumulation (Nisoli *et al*, 1996). In order to further investigate the possible role of β_3 -adrenoceptors in the induction of lipolysis by tumour-derived LMF the effects of SR 59230A upon both lipolysis and adenylate cyclase activation have been studied.

4.1.2 Results

The effects of the β_3 -adrenergic antagonist SR 59230A upon both isoprenaline- and LMF-stimulated lipolysis are shown in Figure 4.1.1. Low concentrations (10^{-9} M- 10^{-6} M) of SR 59230A significantly potentiated LMF-, but not isoprenaline-, stimulated lipolysis in isolated intact white adipocytes, whilst higher concentrations (10^{-5} M) proved inhibitory.

The effect of the β_3 -adrenergic antagonist SR 59230A on isoprenaline and LMF stimulated lipolysis

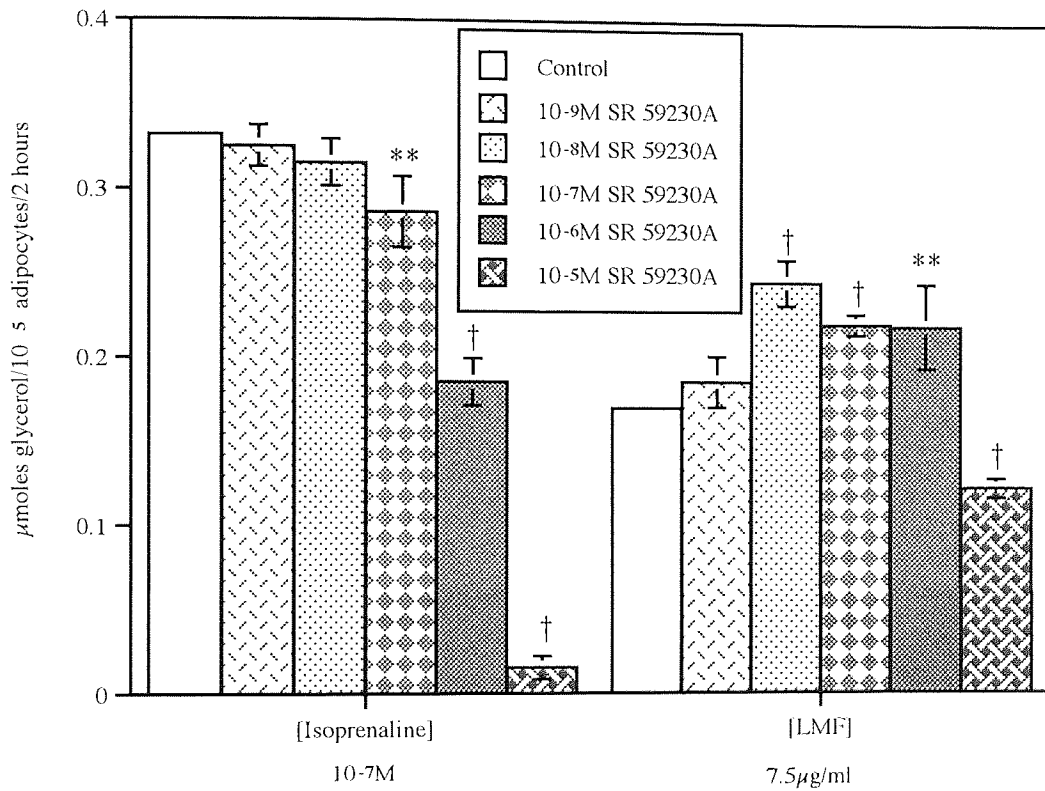


Figure 4.1.1 SR 59230A (10^{-4} M) was dissolved in Krebs Ringer bicarbonate buffer (Section 2.3.1). Adipocytes were separated from non-adipocyte material by washing with SR 59230A supplemented Krebs buffer. In addition, the required concentration of SR 59230A was added to each 1ml aliquot of cells (1×10^5 adipocytes/ml) and pre-incubated for 15 min prior to the addition of the stimulus. Results expressed as mean \pm SEM, where $n=3$. Statistical significance measured using Student's t Test, compared to control, where ** $p \leq 0.05$, † $p \leq 0.005$.

The effect of SR 59230A upon isoprenaline and LMF stimulated adenylate cyclase activity

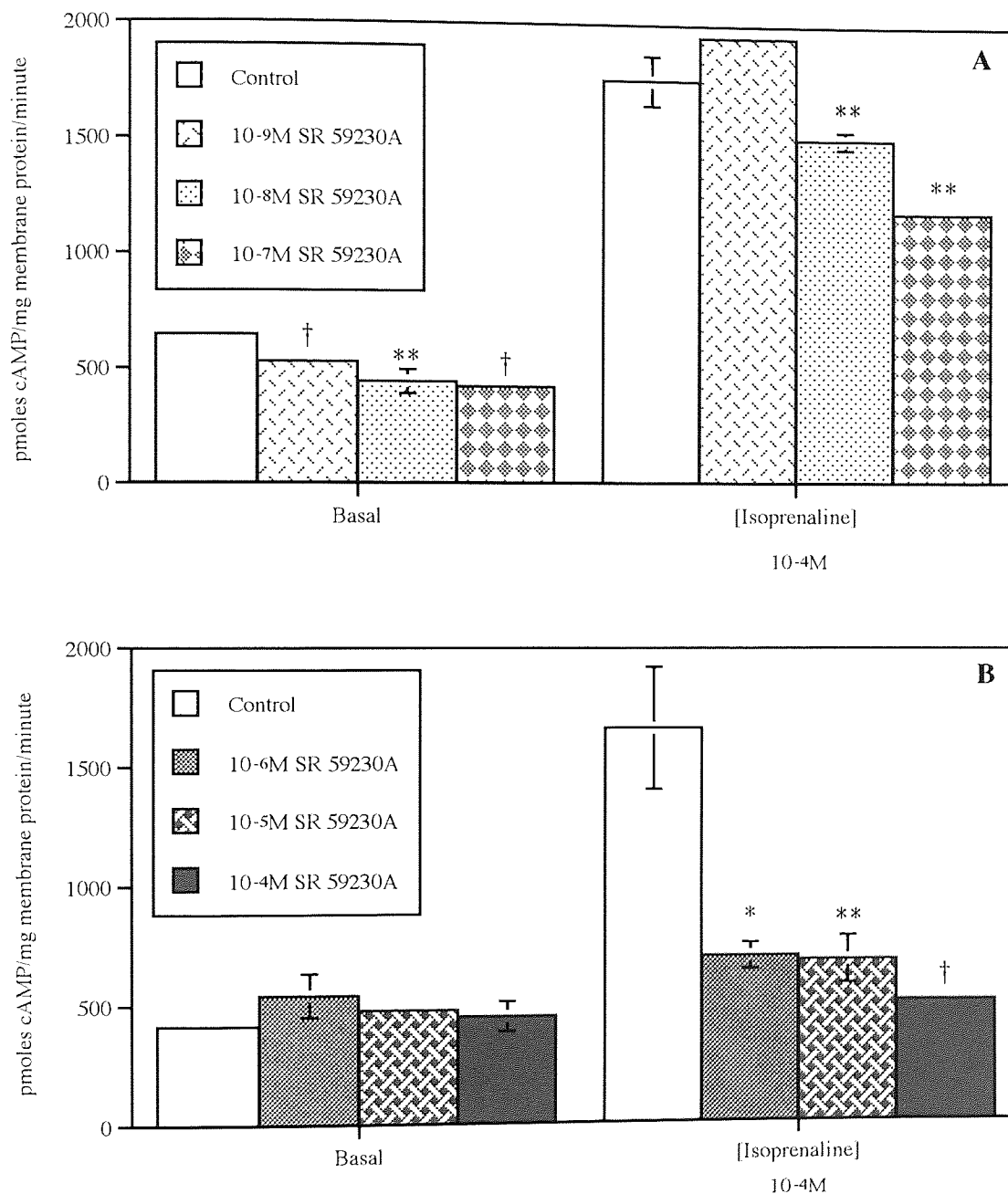
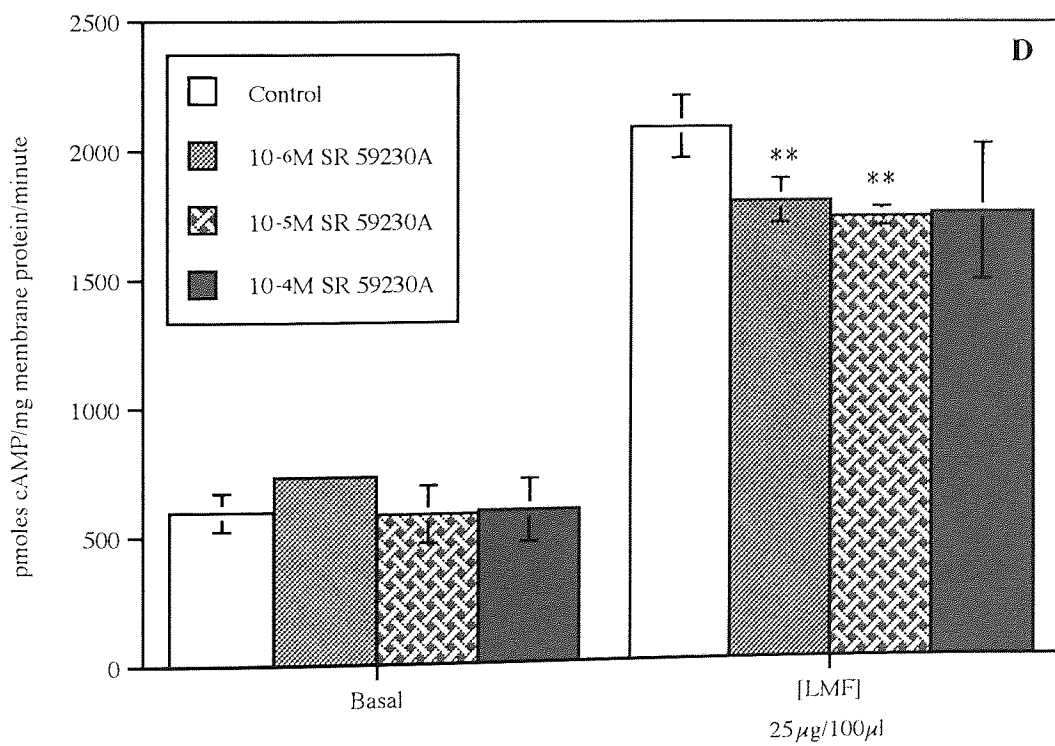
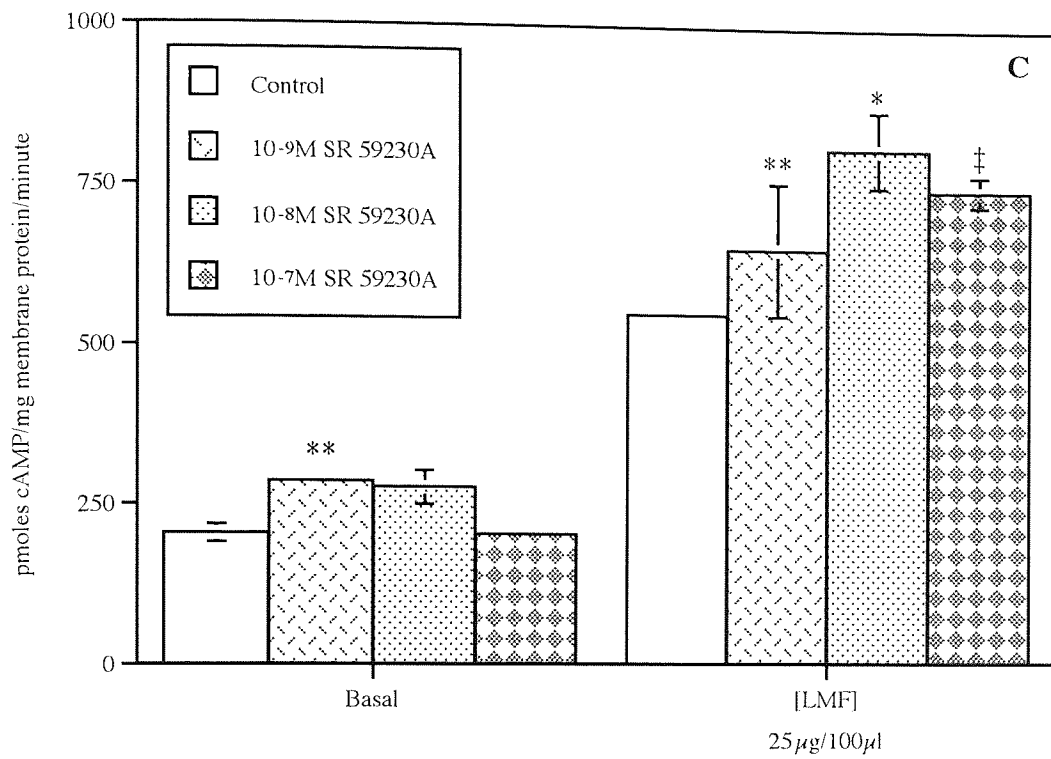


Figure 4.1.2 SR 59230A was dissolved in distilled water (10^{-3} M) and added to isolated adipocyte membranes (typically $30\mu\text{g}/\text{sample}$) which were then pre-incubated for 15 min prior to addition to the incubation mixture. **A** and **B** show the effects of SR 59230A upon isoprenaline stimulated adenylate cyclase activity, **C** and **D** the effects upon adenylate cyclase stimulation by superdex purified LMF. Results expressed as mean \pm SEM where $n=3$. Statistical significance calculated using Student's t Test, compared to control, where $**p\leq 0.05$, $*p\leq 0.01$, $\dagger p\leq 0.005$ and $\ddagger p\leq 0.001$.



The inhibition of isoprenaline-stimulated lipolysis by SR 59230A was more profound than the inhibition of LMF-stimulated lipolysis. However, 10^{-5} M SR 59230A significantly attenuated the induction of lipolysis in isolated adipocytes by both isoprenaline and LMF. Similarly, pre-treatment of adipocyte plasma membranes with SR 59230A (10^{-8} M- 10^{-4} M) produced a significant inhibition of isoprenaline-induced adenylate cyclase activity (Figure 4.1.2A and B). Conversely, as demonstrated in Figure 4.1.2C and D, 10^{-9} - 10^{-7} M SR 59230A significantly potentiated LMF-stimulated adenylate cyclase activity, whilst higher concentrations (10^{-6} M- 10^{-4} M) proved inhibitory.

4.1.3 Discussion

The nature of the receptor involved in the signal transduction pathway associated with the MAC16 tumour-derived LMF has been the subject of much investigation. Khan (1996) demonstrated a significant inhibition of the effects of LMF by the addition of either 100μ M or 10μ M of the non-selective, competitive β -adrenergic antagonist propranolol, thus implying a role for a β -adrenoceptor in the mediation of LMF-induced lipolysis. Further studies, revealed an up-regulation, rather than the expected down-regulation, of adipocyte responsiveness in cells isolated from cachectic MAC16 tumour-bearing mice. This observation, allied with the knowledge that unlike β_1 - and β_2 -adrenoceptors the β_3 -adrenoceptor may lack the structural determinants involved in agonist-induced receptor desensitisation (Giacobino, 1995), lead Khan to postulate that the β -adrenoceptor in question may be an atypical, or β_3 -adrenoceptor. The results shown in Figures 4.1.1 and 4.1.2 would appear to support this hypothesis, with a significant inhibition of both isoprenaline and LMF stimulated lipolysis and adenylate cyclase activity occurring upon pre-treatment of either intact adipocytes, or plasma membrane fractions, with SR 59230A.

The β_3 -adrenoceptor has been proposed to be the predominant lipolytic receptor in most animal species, and has been studied as a potential target for anti-obesity and anti-diabetes drugs due to its almost exclusive distribution in white and brown fat, and its relative ability to withstand desensitisation (Arner, 1996). The presence of functional β_3 -adrenergic

receptors in human adipose tissue, however, remains controversial. Deng *et al* (1996) were unable to detect the presence of β_3 -adrenoceptor mRNA in human omental white adipose tissue, reporting that β_1 - and β_2 -adrenoceptor mRNAs composed 9% and 91% of total β -adrenoceptor mRNA. β_3 -adrenoceptors have, however, been reported in human subcutaneous white adipocytes, but their contribution to the control of lipolysis has been judged to be minimal (Barbe *et al*, 1996; Tavernier *et al*, 1996). Interestingly, Portillo *et al* (1995) reported the presence of β_3 -adrenoceptors in human omental and retroperitoneal adipocytes, but they also recorded a low induction of lipolysis by the β_3 -adrenergic agonist BRL 37344 ((RR) - \pm - 4 [2' - {2 - hydroxy - 2 - (3 - chlorophenyl) ethylamino} propyl] phenoxyacetate), and suggested that this indicated that human adipocytes are poorly equipped with functional β_3 -adrenoceptors. In contrast, in a review of several individual studies examining the physiological functions of the human β_3 -adrenoceptor, Emorine *et al* (1994) concluded that whilst the β_3 -adrenoceptor may not, under normal circumstances, be as important in the control of fatty acid mobilisation in man as it is in rodents, functional receptors do appear to be present in man.

Therefore, whilst it would appear that LMF is unlikely to interact with either β_1 - or β_2 -adrenoceptors, since adipocytes from cachectic mice exhibited enhanced rather than attenuated responsiveness to LMF, the controversy surrounding the presence or absence of β_3 -adrenoceptors in man, allied with reports of relatively low lipolytic responses to selective β_3 -adrenergic agonists, makes the role of LMF in clinical cancer cachexia indistinct. The paucity of β_3 -adrenoceptors in man would appear to imply that either LMF plays a minimal role in the progression of cachexia, or that LMF is actually interacting with either a different class of β -adrenoceptor or an entirely novel receptor. Further receptor binding studies are required in order to conclusively ascertain the nature of the receptor involved in the activation of lipolysis by LMF. Furthermore, the effects of LMF upon the induction of lipolysis and adenylate cyclase activity in human adipocytes require clarification. Thus, at present, of all the β -adrenergic receptors which have been classified it would appear that the β_3 -adrenoceptor is the most likely candidate for the initial stage of

the induction of lipolysis by LMF. However, until more conclusive evidence of direct binding of LMF to β_3 -adrenoceptors has been established, the involvement of a novel, perhaps β_4 -adrenergic, receptor cannot be excluded.

4.2 Comparison of the Responsiveness of Murine and Human White Adipocytes to Murine and Human Lipid-Mobilising Factors

4.2.1 Introduction

The abilities of both MAC16 tumour-derived (Beck and Tisdale, 1987) and cancer patient-derived (Groundwater *et al*, 1990) lipid-mobilising factor (LMF) to induce lipolysis in isolated murine white adipocytes have been well documented. In contrast, the effect of LMF upon human white adipocytes has not been investigated. Thus, whilst it has been postulated that a single species of LMF contributes to the loss of host adipose tissue occurring in both experimental models of cachexia, and clinical cancer cachexia, there is no conclusive evidence that either murine or human LMF possesses the capability to mobilise lipid stores in humans. Indeed, considering the results demonstrated in Chapter 4.1, and suggestions of the relative paucity of β_3 -adrenoceptors in human adipose tissue (Lafontan and Berlan, 1993; Giacobino, 1995) it may be surmised that LMF will not stimulate lipolysis in human adipocytes, and therefore, cannot be involved in the mediation of cancer cachexia. The comparative effects of the β -adrenergic agonist isoprenaline, murine LMF and human LMF in both murine and human white adipocytes have been investigated in an attempt to ascertain further the exact role of LMF in the development and progression of clinical cancer cachexia.

4.2.2 Results

The relative responsiveness of human and murine adipocytes to β -adrenergic stimulus is illustrated by Figure 4.2.1. At low concentrations of isoprenaline ($0M-10^{-9}M$) significant lipolysis occurred in neither human nor murine adipocytes. Higher concentrations, however, ($10^{-8}M-10^{-4}M$) induced a marked dose-dependent release of glycerol from murine adipocytes, with the maximal response occurring with $10^{-5}M$ isoprenaline. Human adipocytes also responded to higher isoprenaline concentrations, however, this response was approximately one third of that demonstrated by murine adipocytes. Thus, murine adipocytes would appear to be more sensitive to β -adrenergic stimulus than their human

Relative responsiveness of human and murine white adipocytes to the β -adrenergic agonist isoprenaline

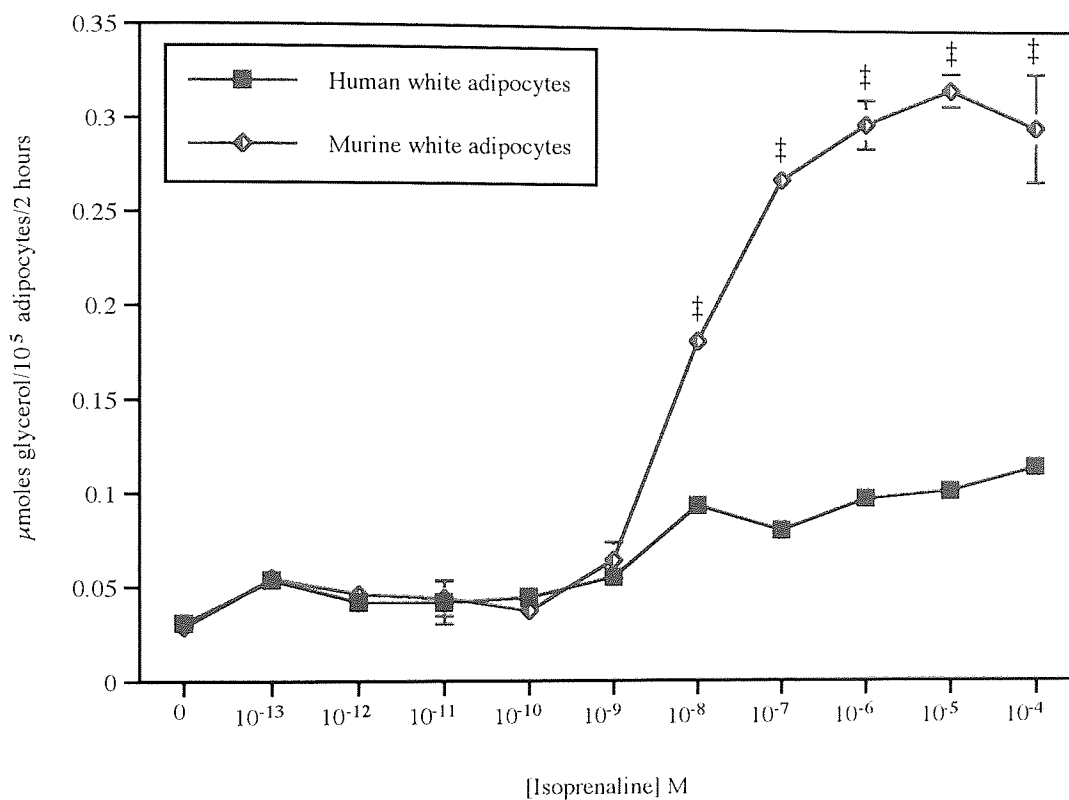


Figure 4.2.1 The β -adrenergic agonist isoprenaline was dissolved (10^{-3} M) in distilled water. Human omental adipocytes and murine epididymal adipocytes (both 1×10^5 cells/ml) were incubated in 1ml aliquots with increasing concentrations of isoprenaline. After 2h samples were deproteinised, neutralised and glycerol release measured. Results expressed as mean \pm SEM where $n=3$. Statistical significance measured using Student's t Test, where $\ddagger p \leq 0.001$.

Relative responsiveness of isolated human and murine white adipocytes to MAC16 tumour-derived LMF

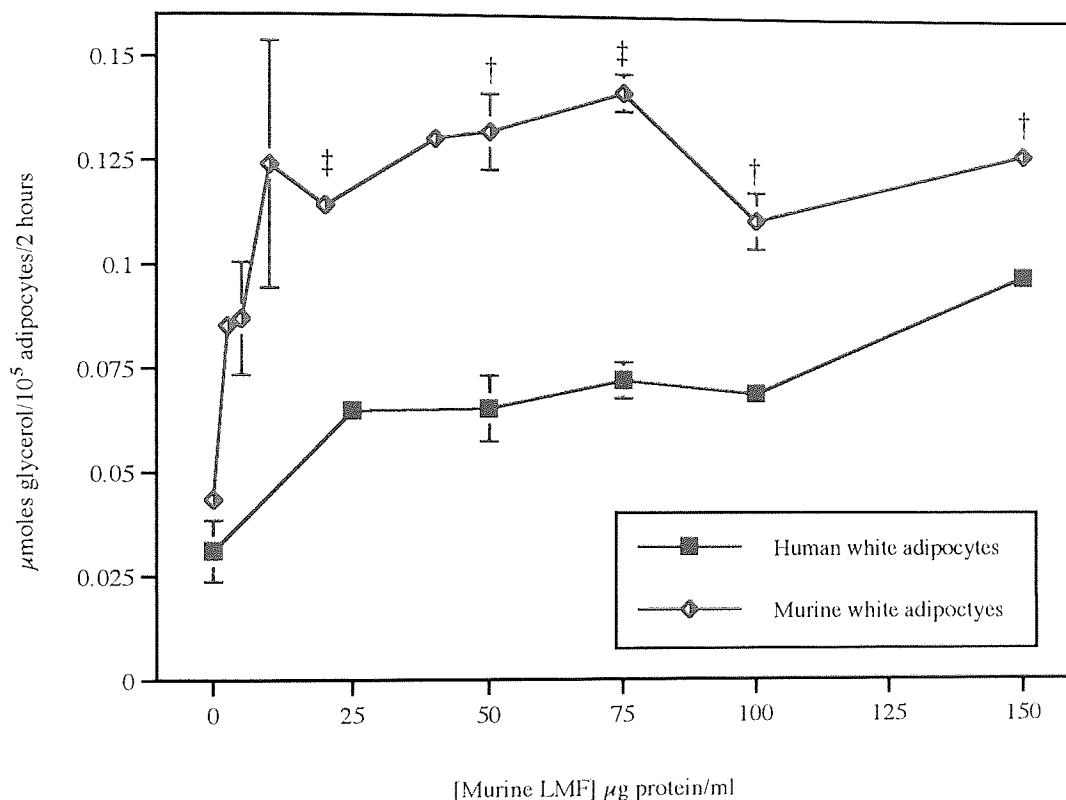


Figure 4.2.2 LMF was purified from MAC16 tumours by sequential batch extraction, anion exchange chromatography and gel exclusion chromatography. Isolated human omental, and murine epididymal white adipocytes (both 1×10^5 cells/ml) were incubated in 1ml aliquots with increasing concentrations of murine LMF for 2h. Incubation supernatants were deproteinised, neutralised and glycerol release measured. Results expressed as mean \pm SEM where $n=3$. Statistical significance measured using Student's t Test, where $\dagger p \leq 0.005$, $\ddagger p \leq 0.001$.

Relative responsiveness of isolated human and murine white adipocytes to human-derived LMF

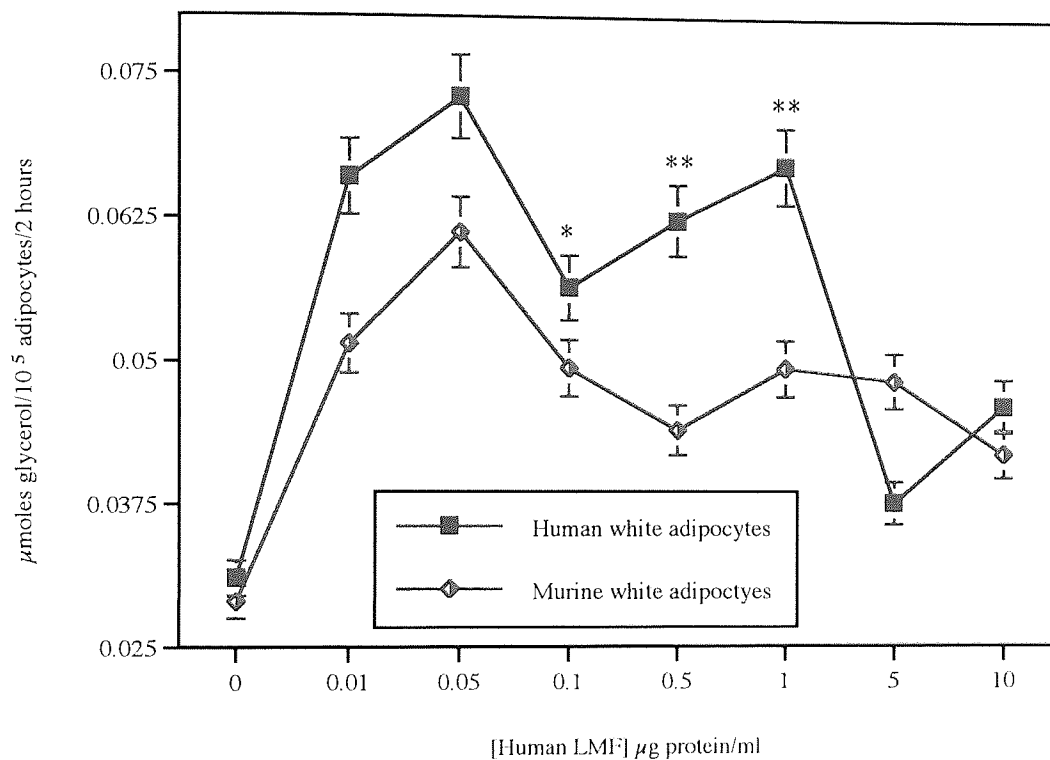


Figure 4.2.3 Human LMF was purified from the urine of cachectic cancer patients via ammonium sulphate precipitation and anion exchange chromatography. Isolated human omental, and murine epididymal white adipocytes (both 1×10^5 cells/ml) were incubated in 1ml aliquots with increasing concentrations of human LMF for 2h. Incubation supernatants were deproteinised and glycerol release measured. Results expressed as mean \pm SEM where $n=3$. Statistical significance measured using Student's t Test, where ** $p \leq 0.05$, * $p \leq 0.01$.

counterparts. Similarly, incubation of murine white adipocytes with murine MAC16 tumour-derived LMF (Figure 4.2.2) induced a significant dose-dependent lipolytic response which was approximately two fold greater than that exhibited by human adipocytes.

The pattern of lipolytic response to murine LMF was similar in both human and murine adipocytes, with 25 μ g/ml significantly stimulating lipid-mobilisation. Interestingly, whilst murine adipocytes demonstrated a greater degree of responsiveness to murine LMF, human adipocytes appeared to be more sensitive to stimulation by human LMF (Figure 4.2.3), with 0.05 μ g/ml inducing the greatest mobilisation of intracellular lipid stores. Although the pattern of lipolytic response demonstrated by fat cells from both species was alike, the actual responsiveness of murine adipocytes to human LMF was approximately three quarters that of human adipocytes. Thus it would appear that the sensitivity of white adipocytes to LMF may be species specific, with greater lipolysis occurring when adipocytes are exposed to LMF derived from the same species.

4.2.3 Discussion

It has been reported that, unlike adipocytes from most other species, human fat cells maintain a high basal lipolytic rate *in vitro*, but exhibit a lower rate of agonist-stimulated lipolysis (Arner, 1996). The human fat cells utilised in this study demonstrated a comparatively low basal lipolytic rate, almost identical to that of murine adipocytes, however, the relative responsiveness of human adipocytes to a non-selective β -adrenergic agonist, isoprenaline, was significantly lower than that observed with murine fat cells. Similarly, when adipocytes were incubated *in vitro* in the presence of a murine tumour-derived LMF, a notably lower stimulation of lipolysis was observed in human fat cells, whilst murine adipocytes exhibited a two to three fold greater degree of sensitivity. Interestingly, however, when LMF purified from the urine of cachectic cancer patients was added to fat cells *in vitro*, human adipocytes demonstrated a significantly greater degree of responsiveness. Thus it is possible that a greater degree of lipolysis would be manifest if

isolated white adipocytes are incubated *in vitro* with LMF derived from the same species. Such an hypothesis could explain the observation of a significantly attenuated stimulation of lipolysis by murine LMF, but not isoprenaline, in rat white adipocytes (Khan, 1996).

Adipose tissue is a heterogeneous organ, and fat deposits from different anatomical sites may demonstrate markedly different responses to lipolytic stimuli (Arner, 1996). Abdominal subcutaneous fat cells have been utilised in a variety of investigations (Kather *et al*, 1985; Mauriege *et al*, 1995; Perea *et al*, 1995; Large *et al*, 1997), however, the lipolytic activity of human adipose tissue has been found to be lowest in the subcutaneous femoral/gluteal region, intermediate in the subcutaneous abdominal region and highest in the visceral, or omental, region (Arner, 1995). Thus, omental adipocytes would appear to be the preferential fat depot for *in vitro* studies into lipolysis in human adipocytes. Omental adipocytes were utilised in this study, hence it may be assumed that the lipolytic responses observed represent the optimal stimulation of lipolysis in human fat cells.

It may be observed, therefore, that both murine and human LMFs are capable of inducing lipolysis in isolated human white adipocytes. As postulated by Arner (1996) a relatively lower rate of lipolysis is manifest in human adipocytes when compared to murine fat cells, however, the enhanced lipolytic activity of human LMF in human adipocytes, and murine LMF in murine adipocytes may suggest some species specific link between the ability of tumour-derived factors to induce host catabolism, and the response of adipose tissue to that factor. Further investigations into the specificity of factors derived from tumours implanted in different species to induce lipolysis in adipocytes from other species are required.

4.3 Conclusions

The results reported in Section 4.1 may appear to imply that LMF would be unlikely to induce lipolysis in human adipocytes due to the relative paucity of β_3 -adrenoceptors existing in human adipose tissue. In contrast, Section 4.2 demonstrates that both murine and human LMF are capable of inducing lipolysis in both murine and human adipocytes. Such findings may seem contradictory, but the increased responsiveness of adipocytes from each species to LMF derived from that same species may clarify matters.

The existence of β_3 -adrenoceptors in humans has been a subject of controversy, however, the presence of β_3 -adrenoceptor mRNA has been detected by Northern blotting in human white (Tavernier *et al*, 1996) and brown (Deng *et al*, 1996) adipocytes, and such receptors have been demonstrated to be fully functional (Hoffstedt *et al*, 1995; Portillo *et al*, 1995; Barbe *et al*, 1996). Human β_3 -adrenoceptors have been documented to display 82% overall homology to murine β_3 -adrenoceptors (Lafontan and Berlan, 1993), thus agonists exhibiting the ability to initiate lipolysis in murine fat cells should, theoretically, prove lipolytic to human fat cells. Interestingly, the β_3 -adrenoceptor has been postulated to be insignificant in the induction of lipolysis in human white adipocytes (Portillo *et al*, 1995; Tavernier *et al*, 1996). However, such studies have utilised the synthetically synthesised β_3 -adrenoceptor agonists such as CGP 12177A, and it is possible that whilst such ligands possess the ability to induce lipolysis in rodent adipocytes, the 18% difference in the amino acid sequence of the human β_3 -adrenoceptor may affect the efficacy of these agents in human fat cells.

The significant inhibition of LMF-induced lipolysis and adenylate cyclase activity demonstrated by SR 59230A (Figures 4.1.1 and 4.1.2) is indicative of a putative role for β_3 -adrenoceptors in the induction of lipolysis by this factor. However, whilst SR 59230A has been proved to be ineffective in antagonising the production of cAMP induced by isoprenaline or noradrenaline in rat brain areas rich in β_1 - or β_2 -adrenoceptors (Nisoli *et al*, 1996), the possibility of an inhibition of the signal transduction cascade associated with

other, as yet undiscovered, β -adrenoceptors cannot be excluded. Therefore, the effects of LMF upon adipocytes may indeed be transduced via β_3 -adrenoceptors, but may conversely involve novel adrenoceptor subtype(s).

Alternatively, whilst the lipolytic response of human adipocytes to isoprenaline and murine LMF may be low, a greater degree of responsiveness to human LMF was observed. It is possible that, despite the apparently homologous nature of LMF purified from either mice or humans, subtle differences may exist in either the sequence or three dimensional structure of these two forms of LMF. Given the 18% lack of homology existing between the murine and human β_3 -adrenoceptors, the existence of unique areas within LMFs from various species may optimise the binding of LMF to the receptor. Full sequence analysis of LMF derived from various cases of clinical cancer cachexia, and experimental tumour models, allied with further studies into the nature of the receptor involved in the initiation of lipolysis should verify, or refute, this hypothesis.

Chapter 5 - Results

Investigation into the Mechanism(s) Underlying the Attenuation of Cancer Cachexia by Eicosapentaenoic Acid

5.1 Introduction

The production of a factor possessing lipid-mobilising activity by the cachexigenic MAC16 colon adenocarcinoma was first reported in 1987 by Beck and Tisdale, who demonstrated an increased rate of FFA release from murine epididymal white adipose tissue (WAT) upon incubation with cell free tumour extracts. Further purification and characterisation of the LMF present in the tumour extract revealed a 40kDa protein (Hirai *et al*, submitted for publication) which induced lipolysis via an increase in intracellular cAMP (Tisdale and Beck, 1991).

Adrenergic agonists, and hormones such as glucagon and ACTH, induce fat cell lipolysis as a consequence of a stimulation of adenylate cyclase (AC) activity (Lafontan and Berlan, 1993; Perea *et al*, 1995). Such stimulation of AC results in the conversion of ATP to cAMP and the subsequent activation of cAMP-dependent protein kinase (PKA) which promotes the conversion of HSL from the inactive to the active state, via the phosphorylation of a serine residue and the translocation of HSL to the lipid droplet. It is this HSL catalysed hydrolysis of TAG into FFA and glycerol which represents the rate limiting step of lipolysis, and anti-lipolytic hormones such as insulin deactivate HSL by dephosphorylation of the serine residue (Lafontan and Berlan, 1993). The signal transduction cascade implicated in the stimulation of lipolysis is outlined in Figure 5.1.

The transfer of the signal induced by the binding of LMF to its receptor is the province of heterotrimeric guanine nucleotide binding proteins (G-proteins). All G-proteins consist of an α -, β - and γ -subunit bound to GDP. Release of GDP and subsequent binding of GTP results in the separation of α_{GTP} and $\beta\gamma$ complex (Figure 5.2), each of which may interact

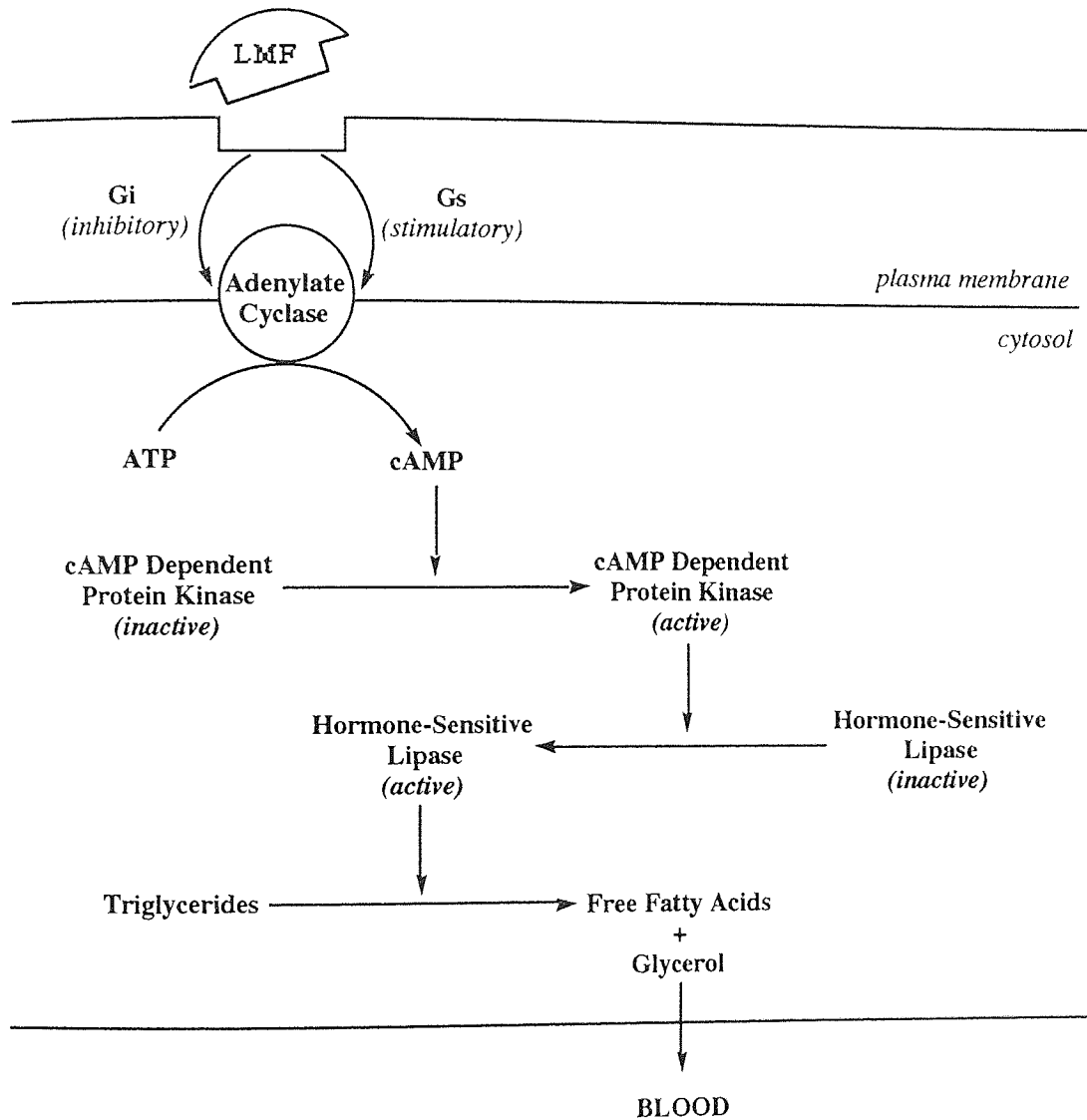
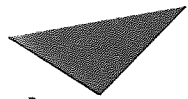


Figure 5.1 The Signal Transduction Cascade Involved in the Induction of Lipolysis by LMF.

with effectors such as AC (Lohse *et al*, 1996). Different cell surface receptors are believed to couple to various patterns of G-proteins, with twenty different mammalian G-protein α -subunits, five β -subunits and ten γ -subunits being, thus far, described (Offermanns and Simon, 1996).



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Figure 5.2 The GTPase Cycle of a Heterotrimeric G-protein. In the lower left hand corner the heterotrimer is depicted in its GDP-bound state. GDP release (1) followed by GTP binding (2) leads to dissociation into the α - and the $\beta\gamma$ -subunits which can now interact with their respective effectors. The α -subunit has an intrinsic GTPase activity (3); when bound GTP is hydrolysed, α - and $\beta\gamma$ -subunits again form the heterotrimer. GDP release is the slowest reaction and is influenced by $\beta\gamma$ -subunits, guanine nucleotide-exchange factors and guanine nucleotide-dissociation inhibitors.

(Taken from Lohse *et al*, 1996)

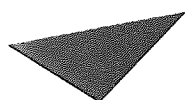
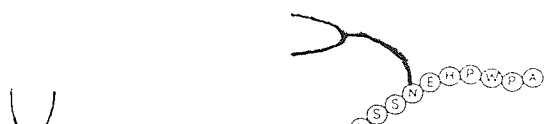
All identified isoforms of AC are known to interact with stimulatory G-protein α -subunits ($G\alpha_s$) to initiate the formation of cAMP (Offermanns and Simon, 1996), and all three β -adrenoceptor subtypes are known to couple to $G\alpha_s$ (Strosberg and Pietri-Rouxel, 1996), hence ligand binding to β_1 -, β_2 -, or β_3 -adrenoceptors results in the phosphorylation and dissociation of $G\alpha_s$ and the activation of AC via $G\alpha_s$ binding. The three β -adrenoceptors which have been thus far characterised demonstrate homology with bacterio-rhodopsin,

consisting of seven sequences of hydrophobic amino acid residues, which correspond to seven transmembrane α -helices, alternately connecting the intracellular and extracellular domains of the receptor (Strosberg, 1996). A schematic representation of the β_3 -adrenoceptor is illustrated in Figure 5.3. In addition to its connections with $G\alpha_s$, it has been postulated that the β_3 -adrenoceptor may also interact with an inhibitory G-protein ($G\alpha_i$) (Strosberg and Pietri-Rouxel, 1996), the activation of which would serve to inhibit the formation of cAMP by AC, and hence impede lipolysis.

The nature of the G-proteins interacting with a receptor may be ascertained via the utilisation of pertussis and cholera toxins (Ribeiro-Neto *et al*, 1985). These toxins are capable of affecting G-protein function via an ADP-ribosylation of the α -subunit, but whilst cholera toxin potentiates $G\alpha_s$ function, thus increasing cAMP levels, pertussis toxin inhibits the action of $G\alpha_i$, thus interrupting the coupling of hormone receptors to $G\alpha_i$ (Ribeiro-Neto *et al*, 1985). Both toxins are hexameric, consisting of a 5-membered oligomer, responsible for the attachment of the toxin to the cell surface, and a sixth subunit capable of ADP-ribosylating bound protein (Ribeiro-Neto *et al*, 1985).

An important pre-requisite for the efficient transduction of receptor generated signals by G-proteins is a close association between the G-protein and the inner surface of the plasma membrane of the cell. Lipid modifications of the $G\alpha$ - and $G\gamma$ -subunits have been reported to be imperative for locating G-proteins alongside other transmembrane, or membrane-associated proteins (Offermanns and Simon, 1996) thus facilitating G-protein functioning. Palmitoylation, the attachment of palmitate (16:0) to a cysteine residue by a thioester bond, is a reversible post-translational modification which may occur to almost all α -subunits, uniting them with the plasma membrane (Hallak *et al*, 1994). The transposable nature of this interaction has been postulated to exhibit a regulatory function, with depalmitoylation of $G\alpha_s$ resulting in the release of the α -subunit to the cytosol (Offermanns and Simon, 1996). Interestingly, $G\alpha_i$ subunits have been demonstrated to be irreversibly bound to myristate (14:0) at an NH_2 terminal glycine residue (Hallak *et al*, 1994). Such irreversible

The Primary Structure of the Human β_3 -adrenoceptor



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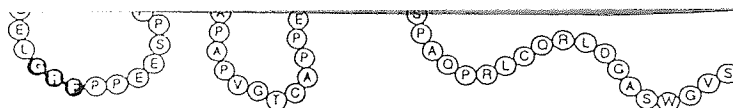


Figure 5.3 Primary structure of the human β_3 -adrenoceptor. The sequences are represented in the one letter code for amino acids (see Appendix 1). The single polypeptide chain is arranged according to the model for rhodopsin. The disulphide bond essential for activity of Cys-111 and Cys-109 is represented by -S-S-. Y-shaped symbols are N-glycosylation sites in the N-terminal of the protein. Wavy vertical line is the palmitoylated Cys-360 residue in the N-terminus of the i4 loop. Residues in black circles are common to all three β -adrenoceptor subtypes.

(Taken from Strosberg, 1996)

myristoylation may contribute to the interaction of α_i with the plasma membrane and $\beta\gamma$ complexes (Offermanns and Simon, 1996).

Fatty acid covalent modification of G-protein α -subunits may, therefore, provide a mechanism whereby certain G-protein-mediated signalling pathways may be curbed. The covalent binding of arachidonate (20:4, n-6), via a thioester linkage, to the G-protein α -subunits present in human platelets was reported by Hallak *et al* (1994), who postulated that the differing physical properties of saturated and unsaturated fatty acids may serve to regulate α -subunit functioning. Furthermore, Muszbek and Laposata (1993) demonstrated the covalent linkage, again involving a thioester bond, of both arachidonate and eicosapentaenoate (20:5, n-3) to the same set of platelet proteins, and suggested that this displacement of a saturated fatty acid by a PUFA may significantly influence the hydrophobic interactions of the protein, and hence have important functional implications.

The administration of a diet rich in fish oil has been demonstrated to significantly alter the fatty acid composition of rat WAT and BAT with replacement of n-6 PUFA by n-3 PUFA (Raclot *et al*, 1994; Leray *et al*, 1995). A similar elevation of EPA and DHA was observed in plasma lipoproteins after fish oil supplementation (Layne *et al*, 1996). Furthermore, Leaf *et al* (1995) reported a significant increase in the concentration of EPA, docosapentaenoic acid (22:5, n-3) (DPA) and DHA present in human adipose tissue following 12 months of dietary n-3 PUFA supplementation. In addition, parenteral administration of n-3 PUFA to patients exhibiting postoperative trauma increased the levels of EPA and DHA present within leukocyte membranes, and enhanced LTC₅ and LTB₅ synthesis (Morlion *et al*, 1996).

Dietary n-3 PUFA administration has also been reported to attenuate the induction of acute inflammation in rats (Nakamura *et al*, 1994), possibly via an inhibition of the 5-LOX pathway in neutrophils and monocytes (Lee *et al*, 1985). Macrophage tumouricidal activity (Erickson and Hubbard, 1996); eicosanoid synthesis (Broughton and Morgan, 1994) and

cytokine production (Purasiri *et al*, 1994) have all been reported to be altered by dietary essential fatty acids, as have expression of major histocompatibility complex class II molecules (Hughes *et al*, 1996), the beating function of the cardiomyocyte (Grynberg *et al*, 1996), the fluidity of keratinocyte membranes and the expression of cell adhesion molecules (Lu *et al*, 1995). Similarly, there is some, but not conclusive evidence that n-3 PUFA may play a role in improving the actions of insulin upon skeletal muscle fibres (Storlien *et al*, 1996).

The mechanisms underlying these many varied actions of fatty acids are multitudinous, though alterations in membrane lipid composition allied with covalent modification of G-proteins may account for some, if not all, of the effects observed. The n-3 PUFA EPA has been noted both to attenuate tumour growth and the progression of experimental cachexia *in vivo*, and to inhibit the induction of lipolysis by a tumour-derived LMF *in vitro* (Tisdale and Beck, 1991). The exact mechanism(s) responsible for this phenomenon are unclear. However, it has been postulated that binding of EPA to $G\alpha_i$ may inhibit the induction of lipolysis, and stimulation of AC activity, by the β -adrenergic agonist isoprenaline (Adamson, 1992). In addition, oral administration of EPA to weight-losing pancreatic cancer patients was noted to attenuate weight loss (Wigmore *et al*, 1996a; Wigmore *et al*, 1996b), possibly via a down-regulation of the APPR associated with cachexia (Wigmore *et al*, 1997c). Furthermore, EPA has been demonstrated to inhibit the growth *in vitro* of human pancreatic cancer cell lines (Falconer *et al*, 1994c), a phenomenon due, at least in part, to cell cycle arrest and the induction of apoptosis (Lai *et al*, 1996).

Interestingly, Connor *et al* (1996) proposed that fatty acids are differentially mobilised from adipose tissue, and that this mobilisation was not proportional to the presence of each fatty acid in adipose tissue, but rather influenced by their molecular structure. Hence, it was noted that EPA and AA demonstrated the greatest rate of mobilisation from adipose tissue TAG to plasma whilst LA and DHA were less readily mobilised. Thus the value of dietary supplementation of weight-losing cancer patients with EPA may appear questionable,

especially if incorporation of this fatty acid into host adipose tissue is followed by its rapid removal due to the catabolic actions of LMF. However, it is probable that orally administered EPA is incorporated not only into adipose tissue TAG but also into adipose tissue plasma membranes, binding covalently to membrane proteins such as AC and G-proteins, and thus being less easily mobilised by catabolic stimuli such as LMF.

In this study, the murine adipocyte was utilised as a model system in an attempt to further clarify the mechanisms involved in the inhibition of tumour-induced lipolysis by the n-3 PUFA EPA. The incorporation of EPA into plasma of patients receiving dietary supplementation with fish oil was ascertained, together with the effects of *in vivo* administration of EPA to either mice and its *in vitro* addition to isolated murine tissues. The effects of EPA on tumour factor production, LMF-induced lipolysis and AC activity were observed and an attempt made to quantify any alterations in adipocyte plasma membrane G-proteins occurring during cachexia.

5.2 Results

5.2.1 Alteration of the Plasma Fatty Acid Profile of Cachectic Pancreatic Cancer Patients upon Oral Administration of Eicosapentaenoic Acid

Oral administration of EPA in the form of either mixed fish oil capsules (MaxEPA), pure EPA capsules, or in a fish oil containing dietary supplement, produced little effect upon the plasma levels of stearic acid (Figure 5.2.1.1), oleic acid (Figure 5.2.1.2), linoleic acid (Figure 5.2.1.3), arachidonic acid (Figure 5.2.1.4) or DHA (Figure 5.2.1.6) in pancreatic cancer patients. However, some patients did demonstrate small, but significant, alterations in plasma fatty acid profile, with a trend for an increased concentration of stearic acid and DHA and a decrease in plasma oleic, linoleic and arachidonic acids. Plasma EPA levels were significantly increased upon supplementation in all patient groups (Figure 5.2.1.5), with barely detectable concentrations prior to treatment increasing to between 3-15% post-supplementation, hence patient compliance to the dietary supplementation regimen has been confirmed.

Alterations in Plasma Stearic Acid Concentration upon Oral Administration of Eicosapentaenoic Acid

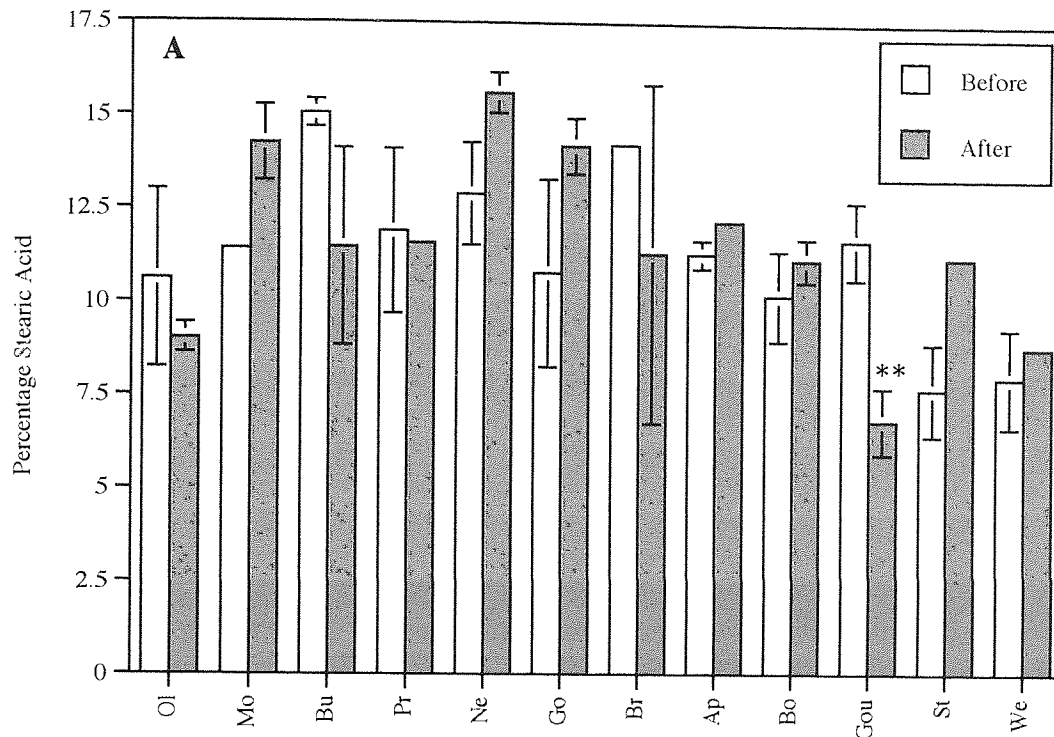
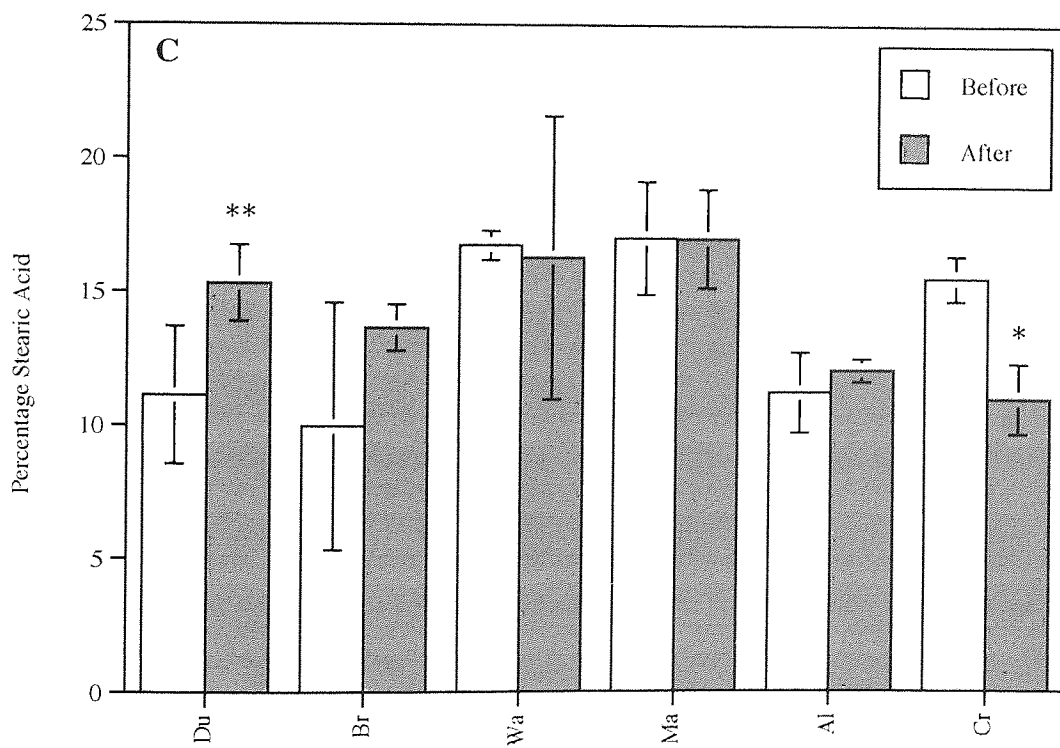
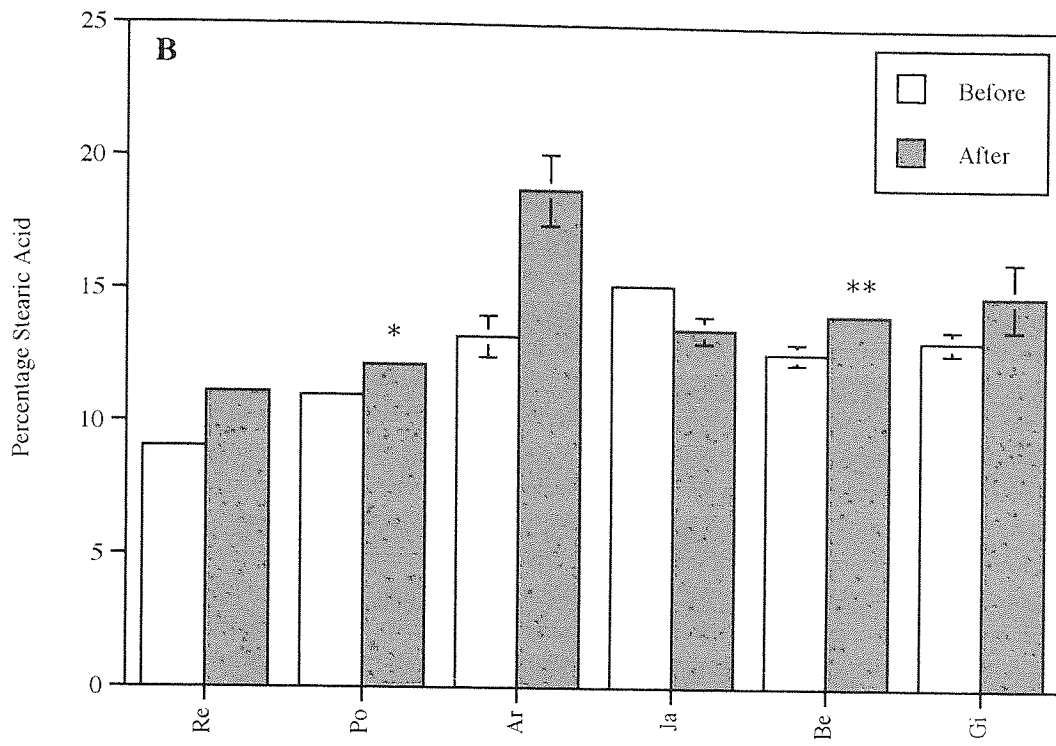


Figure 5.2.1.1 The results illustrate the percentage of total fatty acids present represented by stearic acid. Eicosapentaenoic acid was administered orally to weight-losing advanced pancreatic cancer patients in the form of either MaxEPA capsules (A), 95% pure EPA capsules (B) or a fish oil-containing food supplement (C) (see Section 3.1.1.1). Fatty acids were extracted using the method of Folch *et al* (1957), and the fatty acid methyl esters analysed by capillary gas chromatography. Results expressed as mean \pm SEM, statistical analysis performed using Student's t Test where $n=3$, $**p\leq 0.05$, $*p\leq 0.01$, compared to before supplementation.



Alterations in Plasma Oleic Acid Concentration upon Oral Administration of Eicosapentaenoic Acid

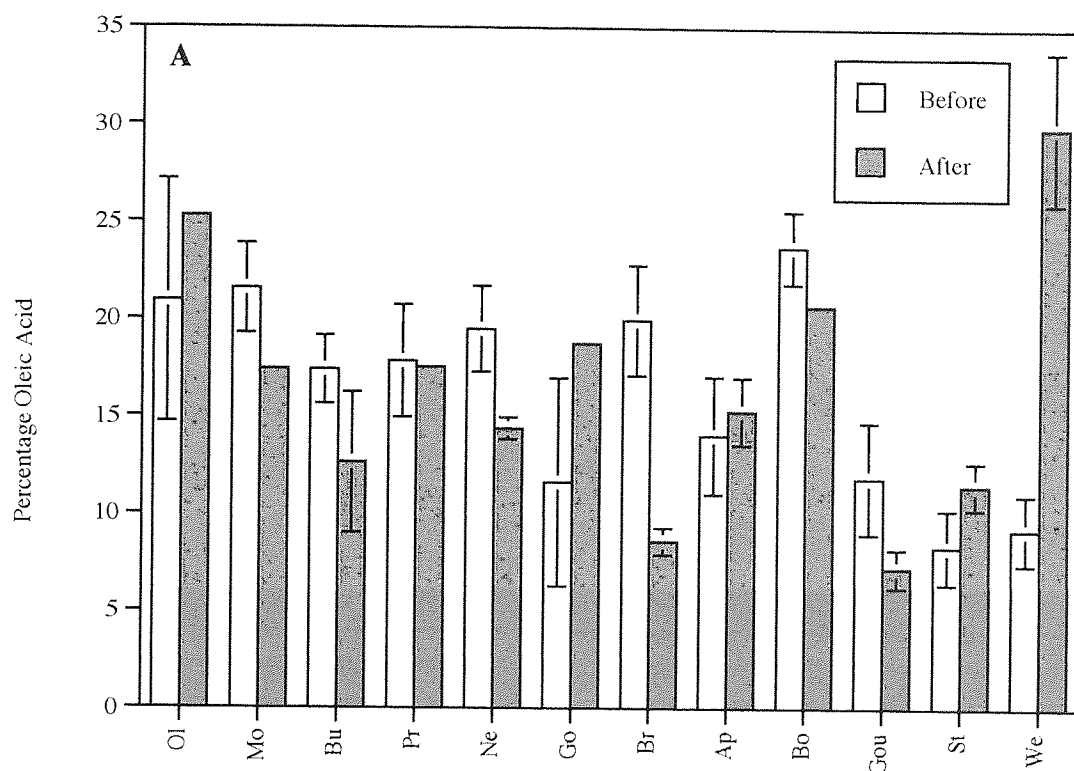
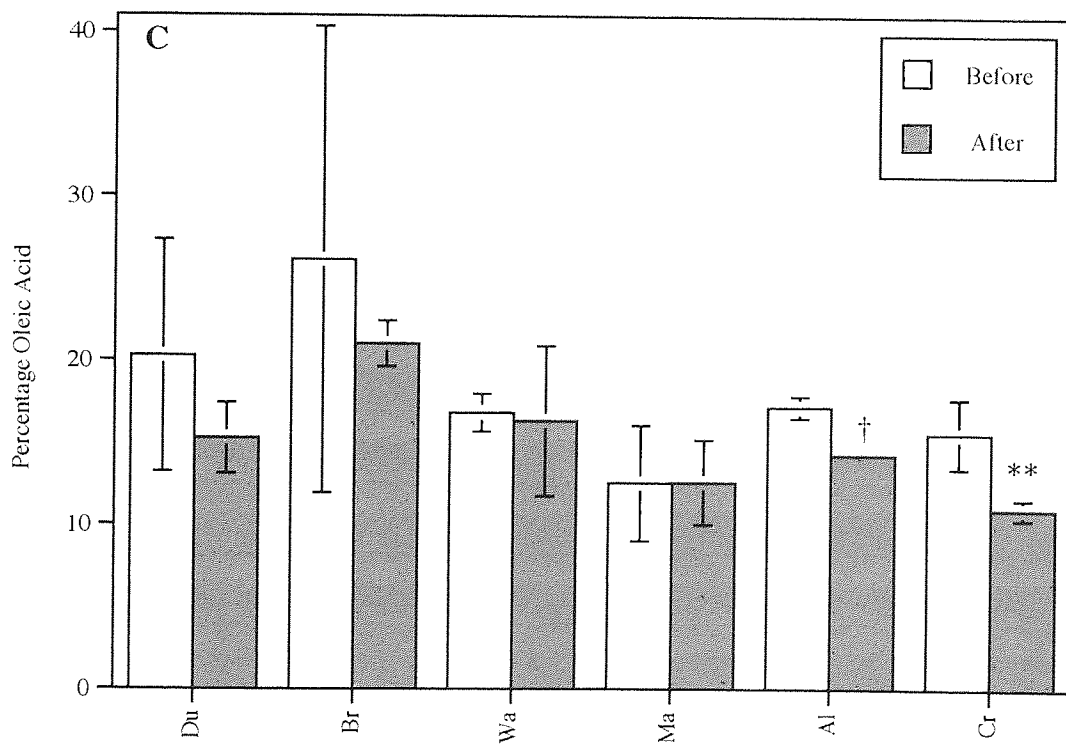
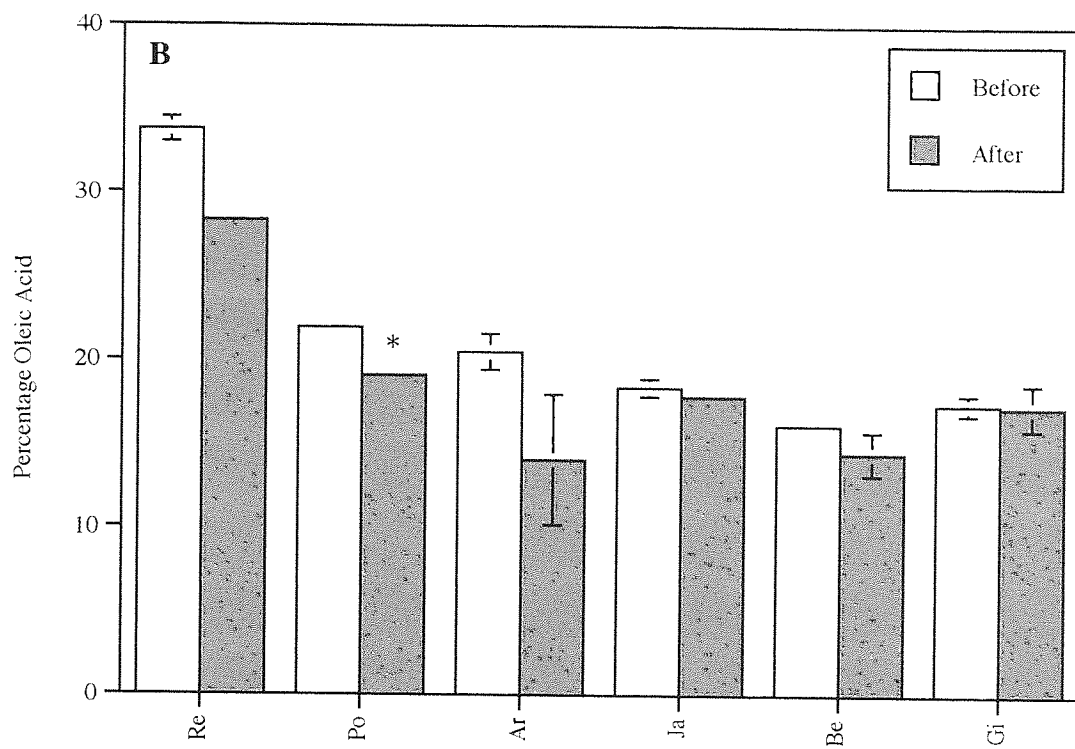


Figure 5.2.1.2 The results illustrate the percentage of total fatty acids present represented by oleic acid. Fatty acids were extracted using the method of Folch *et al* (1957), and the fatty acid methyl esters analysed by capillary gas chromatography. Eicosapentaenoic acid was administered orally to weight-losing advanced pancreatic cancer patients in the form of either MaxEPA capsules (A), 95% pure EPA capsules (B) or a fish oil-containing food supplement (C) (see Section 3.1.1.1). Results expressed as mean \pm SEM, statistical analysis performed using Student's t Test where $n=3$, $**p\leq 0.05$, $*p\leq 0.01$, $\dagger p\leq 0.005$, compared to before supplementation.



Alterations in Plasma Linoleic Acid Concentration upon Oral Administration of Eicosapentaenoic Acid

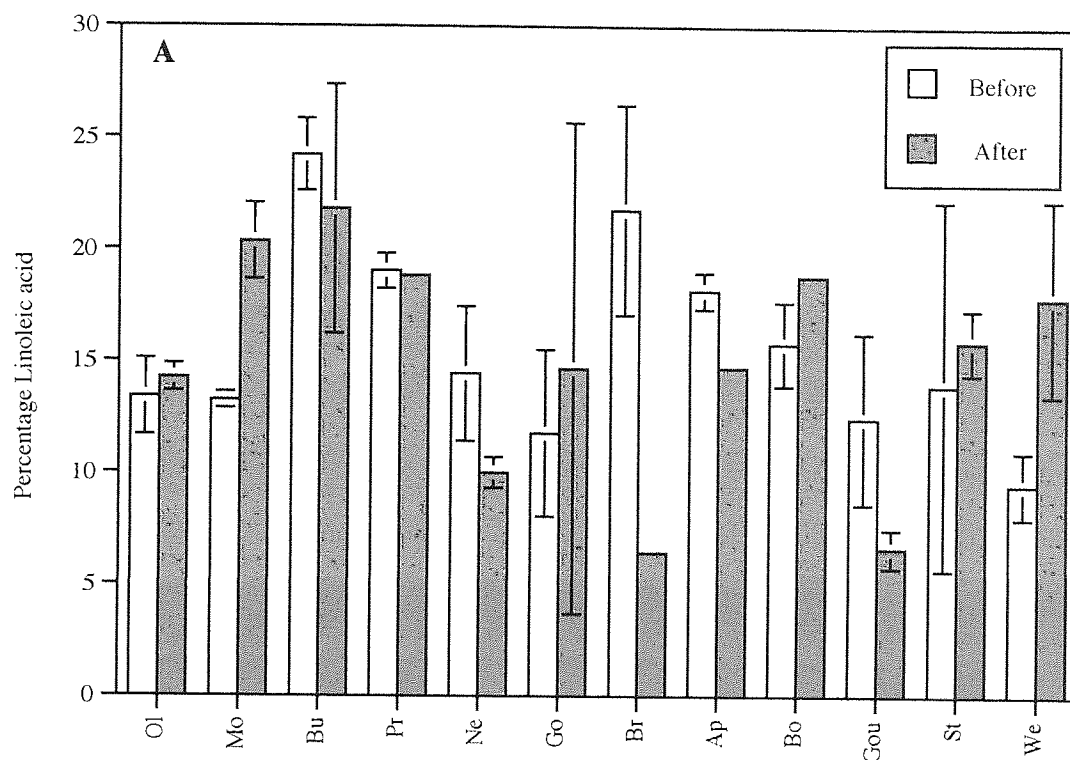
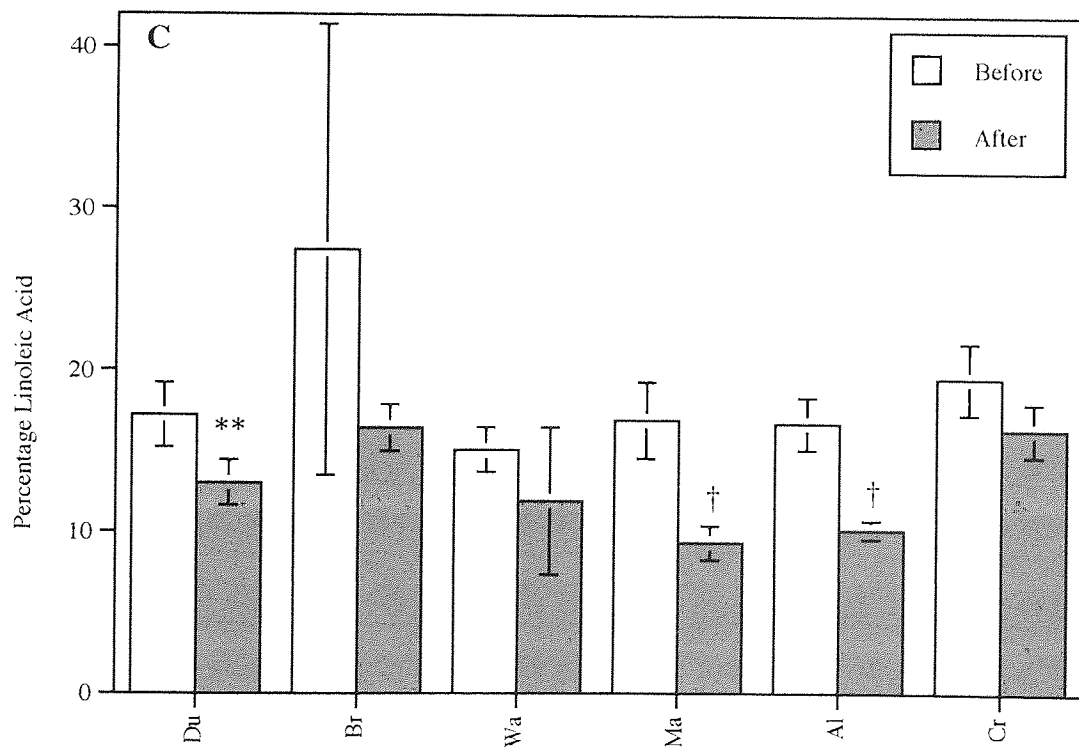
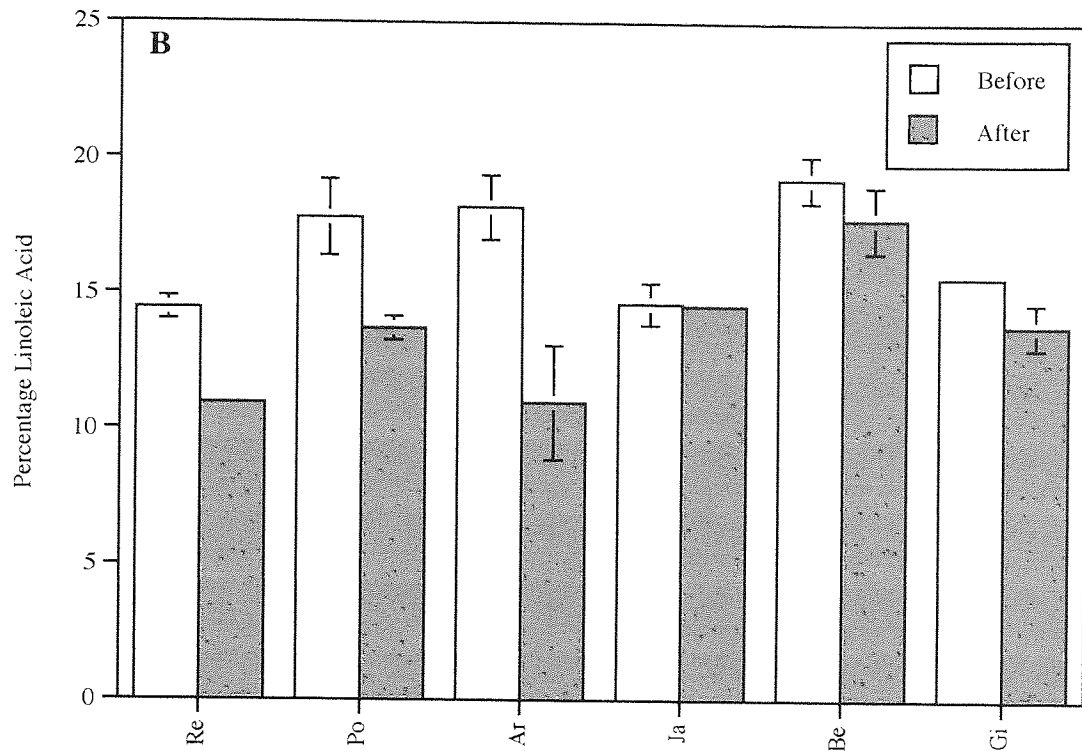


Figure 5.2.1.3 The results illustrate the percentage of total fatty acids present represented by linoleic acid. Fatty acids were extracted using the method of Folch *et al* (1957), and the fatty acid methyl esters analysed by capillary gas chromatography. Eicosapentaenoic acid was administered orally to weight-losing advanced pancreatic cancer patients in the form of either MaxEPA capsules (A), 95% pure EPA capsules (B) or a fish oil-containing food supplement (C) (see Section 3.1.1.1). Results expressed as mean \pm SEM, statistical analysis performed using Student's t Test where $n=3$, $**p\leq 0.05$, $\dagger p\leq 0.005$, compared to before supplementation.



Alterations in Plasma Arachidonic Acid Concentration upon Oral Administration of Eicosapentaenoic Acid

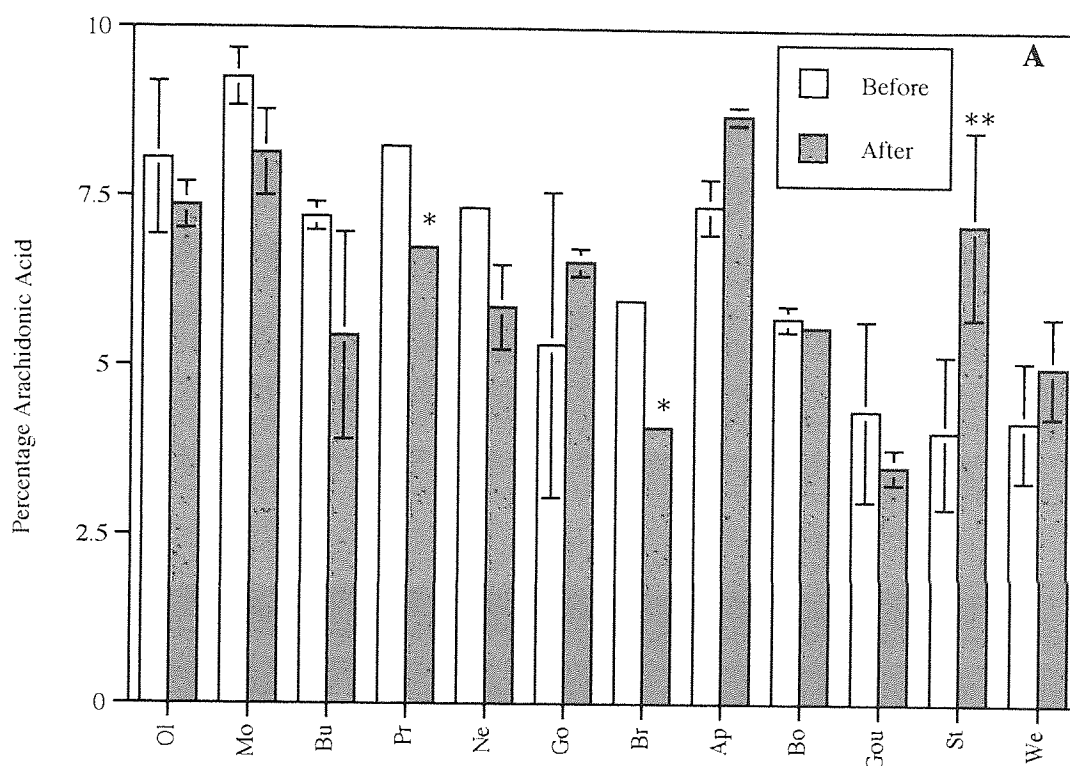
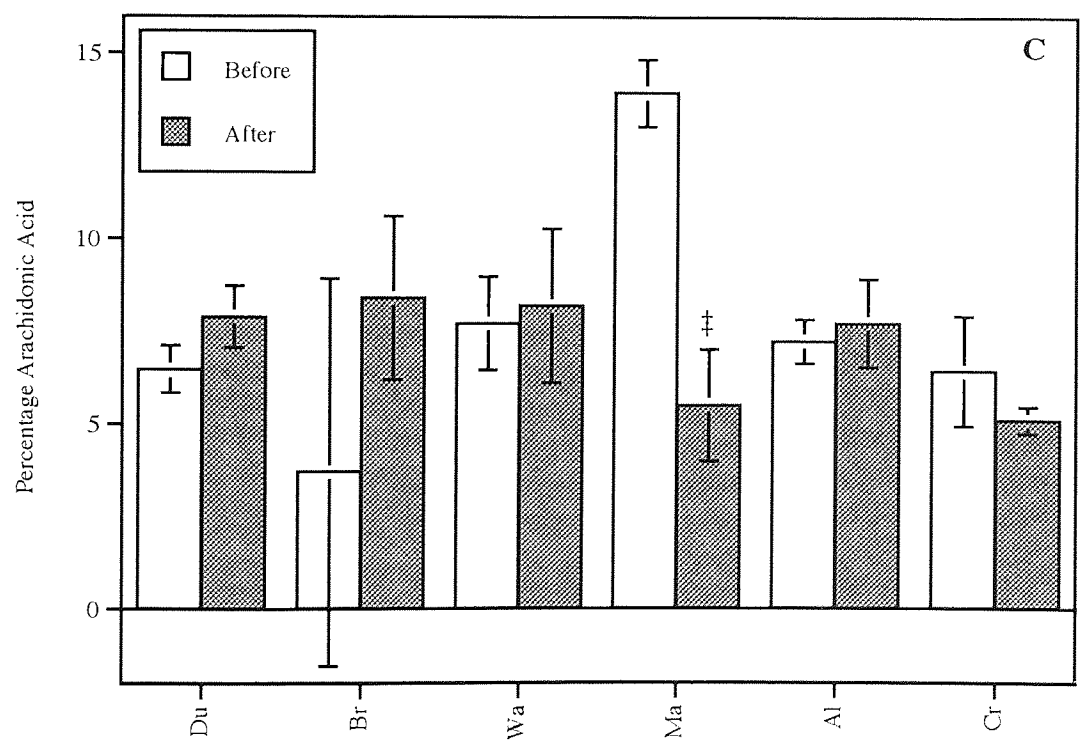
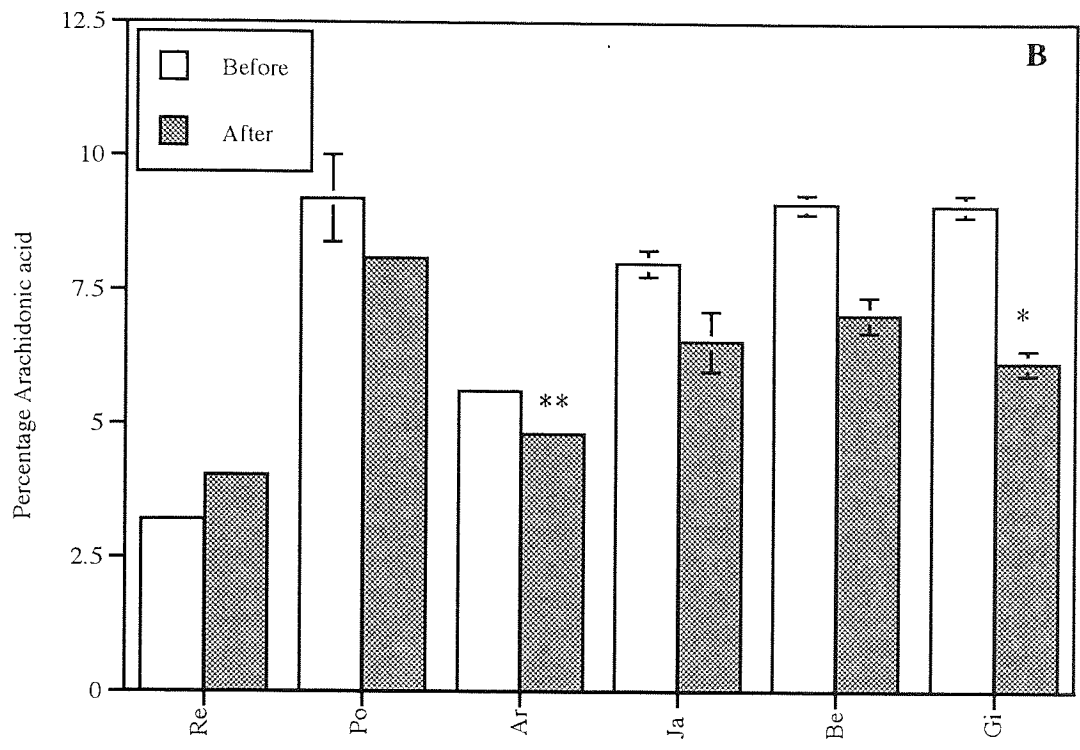


Figure 5.2.1.4 The results illustrate the percentage of total fatty acids present represented by arachidonic acid. Eicosapentaenoic acid was administered orally to weight-losing advanced pancreatic cancer patients in the form of either MaxEPA capsules (A), 95% pure EPA capsules (B) or a fish oil-containing food supplement (C) (see Section 3.1.1.1). Fatty acids were extracted using the method of Folch *et al* (1957), and the fatty acid methyl esters analysed by capillary gas chromatography. Results expressed as mean \pm SEM, statistical analysis performed using Student's t Test where $n=3$, $**p \leq 0.05$, $*p \leq 0.01$, $\ddagger p \leq 0.001$, compared to before supplementation.



Alterations in Plasma Eicosapentaenoic Acid Concentration upon Oral Administration of Eicosapentaenoic Acid

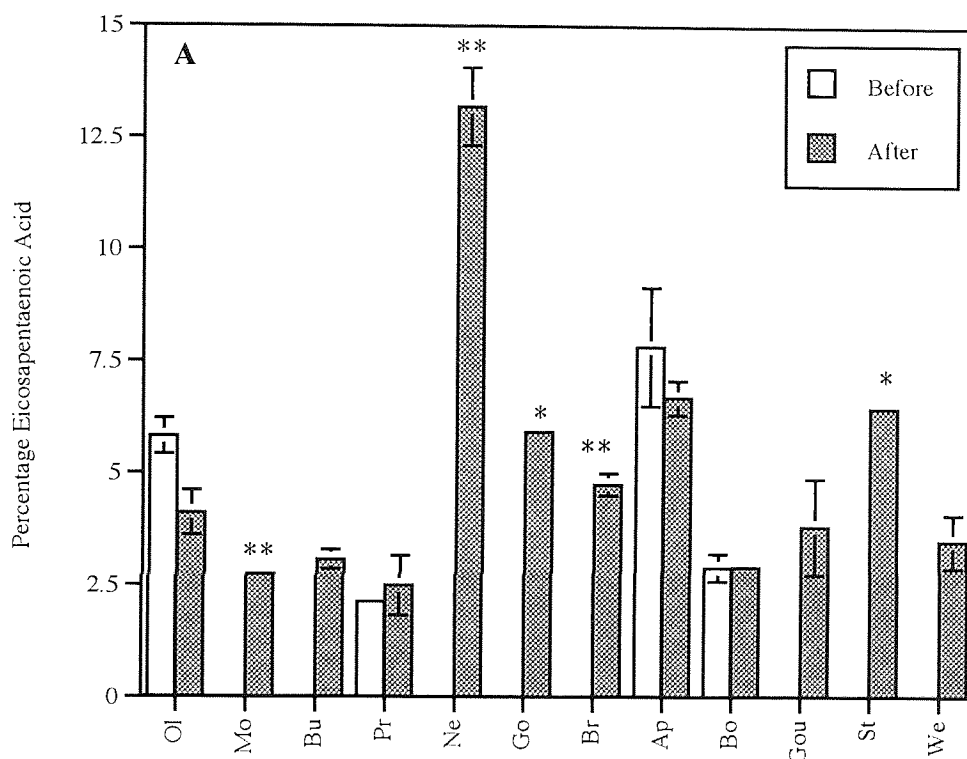
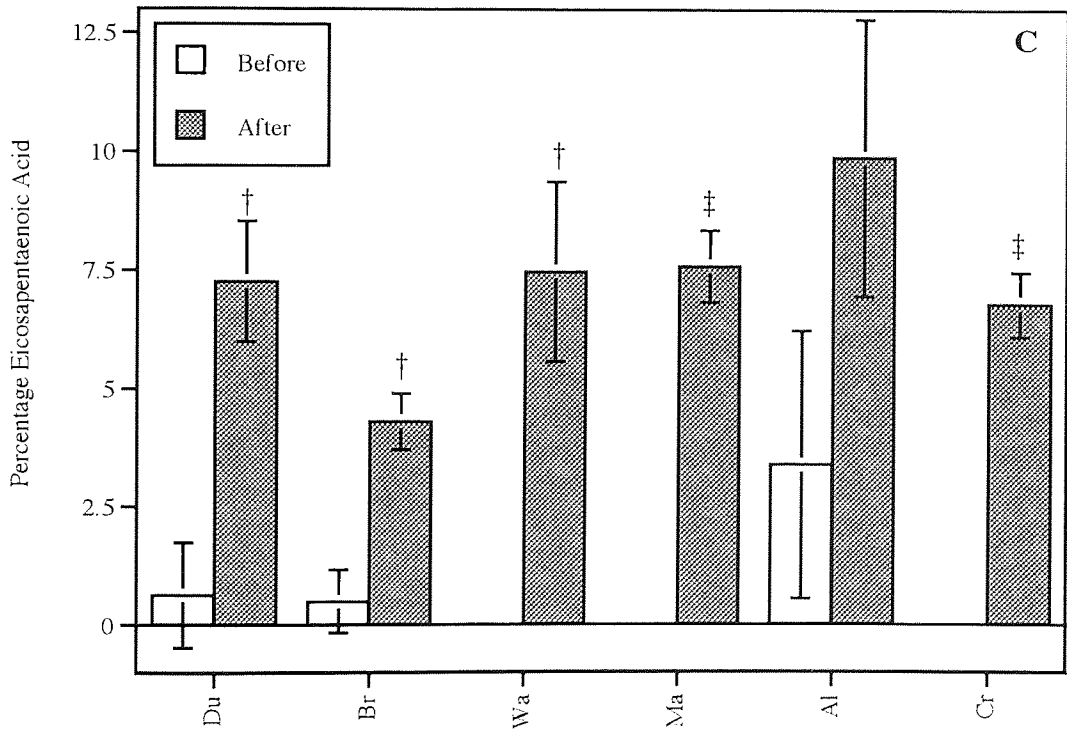
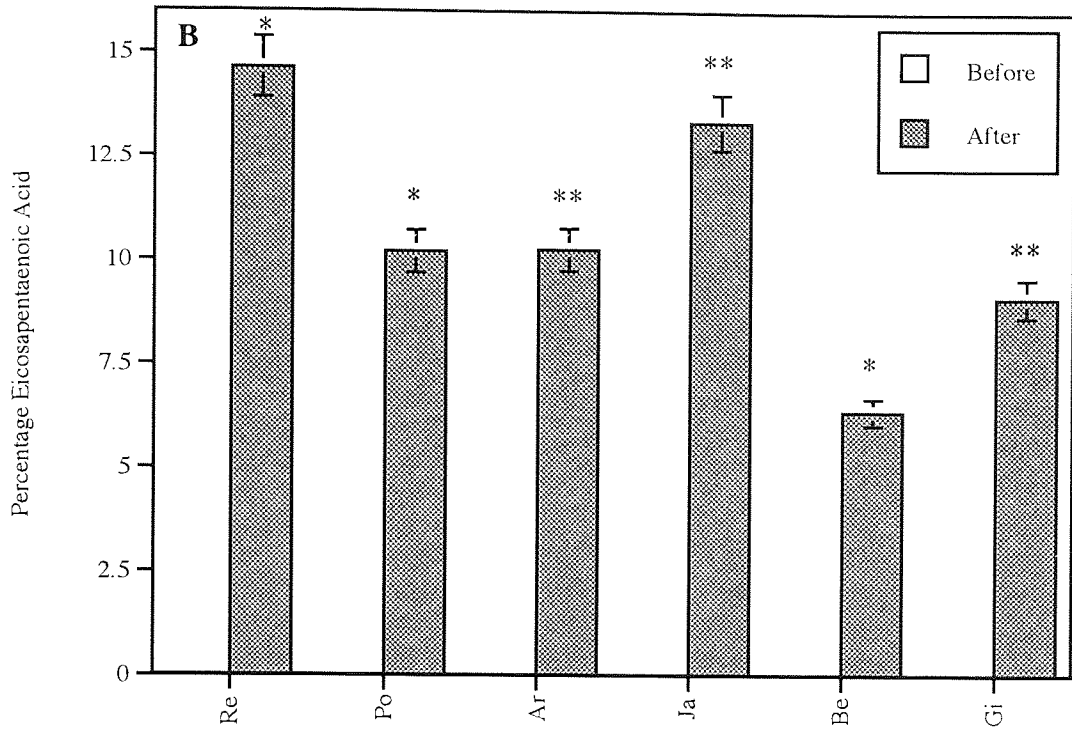


Figure 5.2.1.5 The results illustrate the percentage of total fatty acids present represented by eicosapentaenoic acid. Fatty acids were extracted using the method of Folch *et al* (1957), and the fatty acid methyl esters analysed by capillary gas chromatography. Eicosapentaenoic acid was administered orally to weight-losing advanced pancreatic cancer patients in the form of either MaxEPA capsules (A), 95% pure EPA capsules (B) or a fish oil-containing food supplement (C) (see Section 3.1.1.1). Results expressed as mean \pm SEM, statistical analysis performed using Student's t Test where $n=3$, ** $p \leq 0.05$, * $p \leq 0.01$, † $p \leq 0.005$, ‡ $p \leq 0.001$, compared to before supplementation.



Alterations in Plasma Docosahexaenoic Acid Concentration upon Oral Administration of Eicosapentaenoic Acid

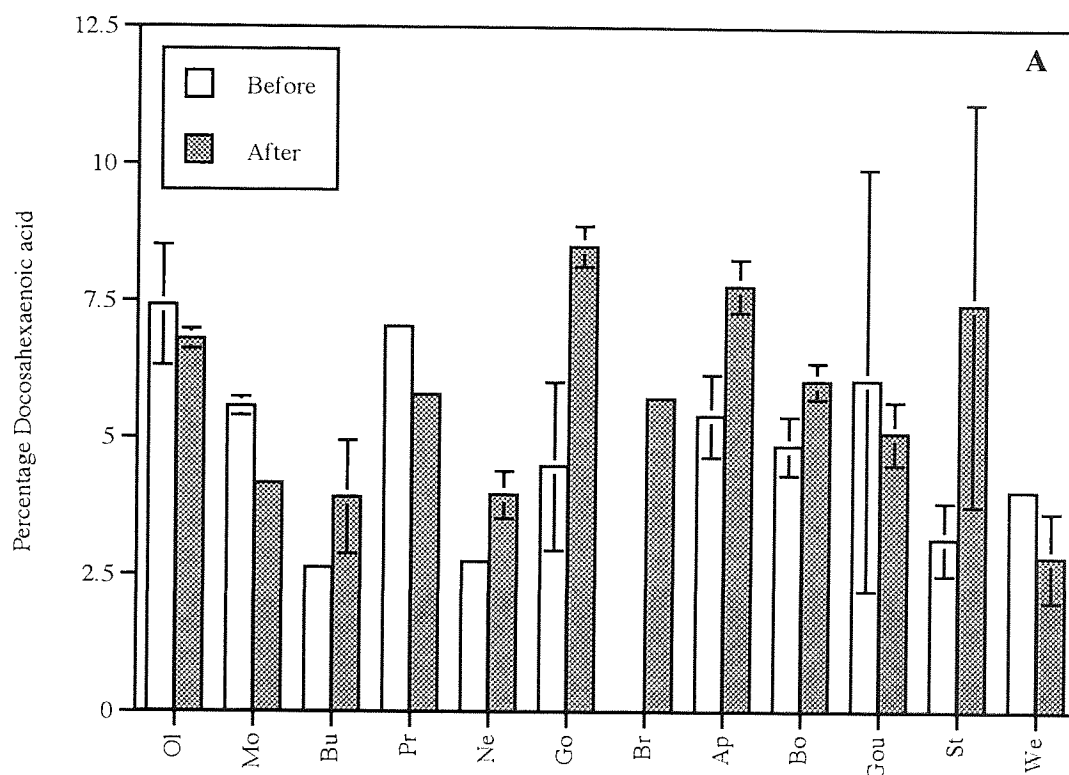
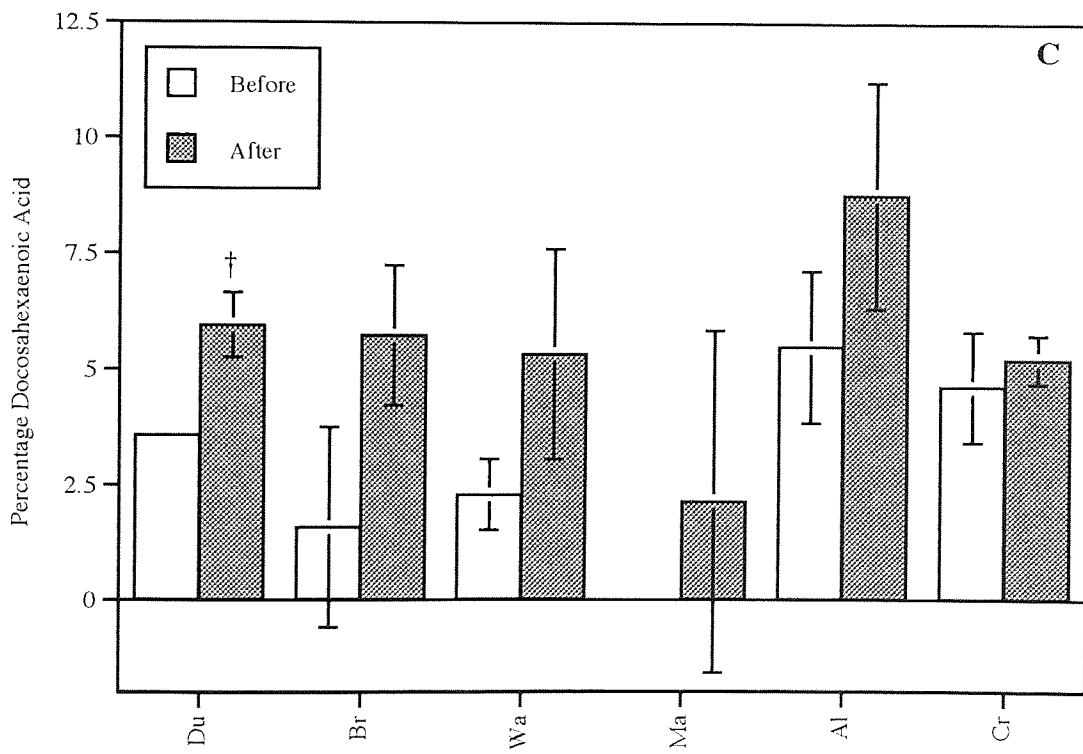
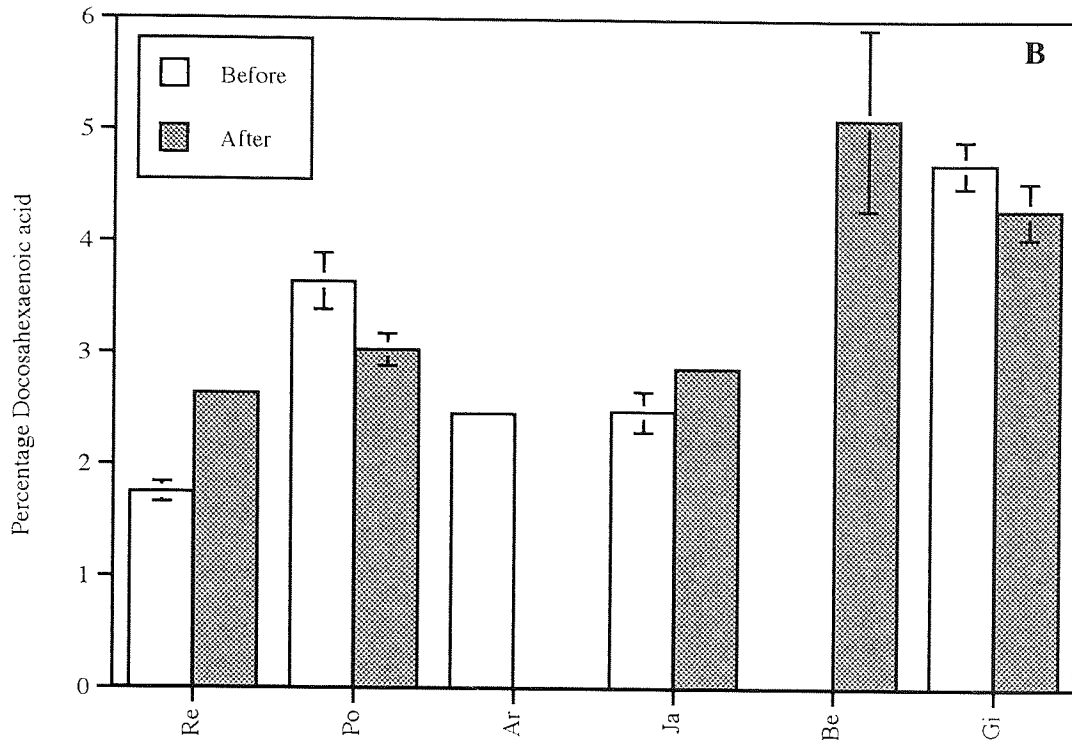


Figure 5.2.1.6 The results illustrate the percentage of total fatty acids present represented by docosahexaenoic acid. Fatty acids were extracted using the method of Folch *et al* (1957), and the fatty acid methyl esters analysed by capillary gas chromatography. Eicosapentaenoic acid was administered orally to weight-losing advanced pancreatic cancer patients in the form of either MaxEPA capsules (A), 95% pure EPA capsules (B) or a fish oil-containing food supplement (C) (see Section 3.1.1.1). Results expressed as mean \pm SEM, statistical analysis performed using Student's t Test where $n=3$, $\dagger p \leq 0.005$, compared to before supplementation.



The degree of incorporation of EPA into patient plasma appeared to correlate with dosage. Patients receiving MaxEPA capsules were supplemented with a maximum of 3g EPA per day, whilst those taking part in the trial of 95% pure EPA capsules were provided with 6g per day. The fish oil-containing dietary supplement also provided patients with a slightly lower dosage of EPA (2g/can, ~4g/day), however, additional calories in the form of both carbohydrates and proteins were also present within this supplement. The greatest increase in plasma EPA concentration was observed in patients receiving pure EPA capsules. In these patients plasma EPA levels increased from undetectable prior to commencement of therapy to an average of $10.66 \pm 2.97\%$ after one month of supplementation. Patients receiving MaxEPA capsules also demonstrated low plasma EPA levels ($1.55 \pm 2.68\%$) which increased significantly after one month of dietary supplementation to an average of $5.00 \pm 2.97\%$. Likewise, the fish oil-containing supplement increased EPA levels from $0.76 \pm 1.32\%$ to $7.21 \pm 1.79\%$ after one month of therapy. Hence it would appear that the increase in plasma EPA levels associated with dietary supplementation with fish oil correlates to dosage, with a two fold greater incorporation of EPA being manifest in patients receiving 95% pure EPA in comparison to those receiving mixed fish oil preparations. In addition, the consumption of two cans per day of the fish oil containing dietary supplement provided a slightly higher dose of EPA than that achieved using MaxEPA capsules (4g/day and 3g/day, respectively), and this was reflected by a 2% greater average incorporation of EPA into plasma, further demonstrating the relationship between dose and degree of incorporation of the fatty acid.

5.2.2 The Effect of Oral Administration of Eicosapentaenoic Acid to Male NMRI Mice upon Tissue Fatty Acid Composition

Oral administration of a single dose of 95% pure EPA (1.25g/kg) to male NMRI mice resulted in a significant increase in serum EPA levels after 24h (from $0.69 \pm 0.98\%$ to $6.70 \pm 2.28\%$) (Table 5.2.2.1). Significant incorporation of EPA could also be demonstrated to occur in murine WAT (from $0.62 \pm 0.07\%$ to $3.69 \pm 0.03\%$) over 24h (Table 5.2.2.2), indicating a rapid turnover of fatty acids within this tissue. In addition, WAT stearic acid levels also increased whilst there was a concomitant decrease in WAT

Incorporation of Orally Administered Eicosapentaenoic Acid into Murine Serum

Fatty acid	Undosed Control (n=3)	1.25g/kg EPA, 24h (n=4)
Stearic	13.35 ± 3.97	15.76 ± 0.87
Oleic	7.07 ± 2.46	9.42 ± 0.71
Linoleic	19.52 ± 7.35	19.74 ± 1.02
Arachidonic	8.37 ± 2.86	8.14 ± 0.21
Eicosapentaenoic	0.69 ± 0.98	6.70 ± 2.28 **
Docosahexaenoic	8.18 ± 2.30	10.00 ± 0.74

Table 5.2.2.1 A single 1.25g/kg dose of 95% EPA was administered post orally to male BKW mice. After 24h blood was removed by cardiac puncture and centrifuged to separate red blood cells from serum. Fatty acids were extracted and analysed as previously described (Sections 3.1.2 and 3.1.3). Results expressed as mean ± SEM, where each fatty acid was calculated as a percentage of total fatty acids present, statistical analysis performed using Student's t Test where **p≤0.05, compared to control.

Incorporation of Orally Administered Eicosapentaenoic Acid into Murine White Adipose Tissue

Fatty acid	Undosed Control (n=3)	24h 1.25g/kg EPA (n=4)
Stearic	3.19 ± 0.42	4.93 ± 0.15 **
Oleic	38.49 ± 0.53	36.32 ± 0.09 **
Linoleic	20.03 ± 0.76	18.94 ± 0.75
Eicosapentaenoic	0.62 ± 0.07	3.69 ± 0.03 ‡

Table 5.2.2.2 A single 1.25g/kg dose of 95% EPA was administered post orally to male BKW mice. After 24h mice were killed by cervical dislocation and epididymal fat pads dissected out. Fatty acids were extracted and analysed as previously described (Sections 3.1.2 and 3.1.3). Results expressed as mean ± SEM, where each fatty acid was calculated as a percentage of total fatty acids present, statistical analysis performed using Student's t Test where **p≤0.05, ‡p≤0.001, compared to control.

Incorporation of Orally Administered Eicosapentaenoic Acid into Murine Gastrocnemius Muscles

Fatty acid	Undosed Control (n=10)	1.25g/kg EPA, 24h (n=6)	1.25g/kg EPA, 48h (n=4)
Stearic	8.03 ± 2.65	12.86 ± 3.86	9.37 ± 0.49
Oleic	24.13 ± 7.38	23.69 ± 5.47	20.89 ± 3.10
Linoleic	12.28 ± 2.08	11.72 ± 1.48	12.62 ± 0.94
Arachidonic	3.51 ± 1.24	3.22 ± 1.66	5.25 ± 0.86 **
Eicosapentaenoic	0.32 ± 0.40	0.15 ± 0.37	3.52 ± 1.01 ‡
Docosahexaenoic	18.67 ± 5.39	19.78 ± 2.94	17.17 ± 0.86

Table 5.2.2.3 A single 1.25g/kg dose of 95% EPA was administered post orally to six male BKW mice, a second dose of EPA was administered to 4 additional mice. After 24h and 48h respectively, mice were killed by cervical dislocation and their gastrocnemius muscles dissected out. Fatty acids were extracted and analysed as previously described (Sections 3.1.2 and 3.1.3). Results expressed as mean ± SEM, where each fatty acid was calculated as a percentage of total fatty acids present, statistical analysis performed using Student's t Test where **p≤0.05, ‡p≤0.001, compared to control.

*In Vitro Incorporation of Exogenous Fatty Acids into Isolated
Adipocyte Plasma Membranes*

Fatty acid	Control	100 μ M EPA	100 μ M DHA	100 μ M AA
Stearic	7.95 \pm 1.31	6.38 \pm 3.38	5.48 \pm 1.47**	5.76 \pm 2.06
Oleic	11.74 \pm 1.89	11.01 \pm 3.19	9.85 \pm 0.87	10.04 \pm 3.33
Linoleic	14.85 \pm 1.32	13.16 \pm 0.90	13.90 \pm 2.26	14.20 \pm 2.64
Arachidonic	5.87 \pm 1.19	6.25 \pm 0.70	4.53 \pm 1.38	3.86 \pm 1.39
EPA	1.22 \pm 2.12	12.32 \pm 5.13**	8.49 \pm 0.52**	9.25 \pm 1.63
DHA	0.00 \pm 0.00	5.98 \pm 2.14**	4.70 \pm 0.50**	9.00 \pm 0.66‡

Table 5.2.3.1 Adipocyte plasma membranes were isolated as previously described using a self-forming Percoll gradient (Section 3.5.1). Membranes (30 μ g) were incubated at room temperature for 30min with 100 μ M EPA, DHA or AA dissolved in ethanol, and then washed three times with sucrose buffer (Section 2.3.7) prior to the extraction, and analysis of fatty acids (Sections 3.1.2 and 3.1.3). Results expressed as mean \pm SEM, where each fatty acid was calculated as a percentage of total fatty acids present. Statistical analysis performed using Student's t Test, where ** $p \leq 0.05$, ‡ $p \leq 0.001$ compared to control.

oleic acid concentration. Conversely, no incorporation of EPA could be observed in skeletal (gastrocnemius) muscle over 24h, however, administration of a second dose of EPA revealed significant ($3.52 \pm 1.01\%$) EPA incorporation after 48h (Table 5.2.2.3), a not unexpected finding considering the slower rate of protein, compared to fatty acid, turnover observed *in vivo*. Interestingly, the concentration of arachidonic acid present within skeletal muscle could also be seen to be significantly greater in muscles taken from 48h dosed mice when compared to undosed and 24h dosed samples. This may however, be merely the consequence of variation between different animals, and may not be an important observation.

5.2.3 In Vitro Incorporation of Fatty Acids into Isolated White Adipocyte Plasma Membranes

The *in vitro* incorporation of fatty acids into isolated white adipocyte plasma membranes was established in order to confirm that alterations in membrane function observed upon incubation with fatty acids were a consequence of an alteration in membrane fatty acid composition. The addition of $100\mu\text{M}$ 99% pure EPA to isolated white adipocyte plasma membranes for 30min resulted in a significant increase in EPA (from $1.22 \pm 2.12\%$ to $12.32 \pm 5.13\%$) and DHA (from 0.00 ± 0.00 to $5.98 \pm 2.14\%$) concentrations, allied with moderately decreased stearic, oleic and linoleic acid levels (Table 5.2.3.1). Similar alterations in membrane fatty acid profile were observed when membranes were pre-incubated for 30min with $100\mu\text{M}$ 99% pure DHA. However, a moderate decrease in AA concentration was also noted. In addition, a significantly greater increase occurred in membrane EPA compared to DHA, indicating retroconversion of this fatty acid to its shorter chain precursor.

Interestingly, however, the addition, for 30min, of $100\mu\text{M}$ 99% pure AA to adipocyte plasma membranes did not result in a significant increase in membrane AA concentration. Rather a moderately decreased level of AA, together with reductions in membrane stearic, oleic and linoleic acids was observed, accompanied by significantly increased membrane EPA and DHA levels (to $9.25 \pm 1.63\%$ and $9.00 \pm 0.66\%$ respectively). At present, there

are no known mechanisms permitting the conversion of an n-6 fatty acid to an n-3 fatty acid. Therefore, it is possible that the AA utilised in this study was impure, perhaps containing EPA or DHA, and so induced increases in membrane levels of these PUFAs.

5.3 Discussion

The incorporation of orally administered PUFAs into plasma lipid fractions (Purasiri *et al*, 1994; Leaf *et al*, 1995; Layne *et al*, 1996), muscle membrane lipids (Grynberg *et al*, 1996; Storlien *et al*, 1996) and WAT and BAT (Leaf *et al*, 1995; Leray *et al*, 1995) has been well documented, as have the beneficial effects of dietary supplementation of weight-losing pancreatic cancer patients with EPA (Wigmore *et al*, 1996a; Wigmore *et al*, 1996b; Wigmore *et al*, 1997c). The incorporation of orally administered EPA into the plasma lipid fractions of these recipient patients has been confirmed, demonstrating patient compliance to the therapeutic regimen.

Leaf *et al* (1995) observed a concomitant incorporation of EPA into plasma lipids and adipose tissue upon oral administration of EPA, suggesting that a similar reciprocal alteration of adipose tissue fatty acid composition may be occurring in pancreatic cancer patients receiving dietary supplementation. Hence alterations in tissue fatty acid composition associated with long term dietary manipulation may be responsible, at least in part, for the attenuation of cancer cachexia observed by Wigmore *et al* (1996a and 1996b).

This hypothesis was supported by the observation that short term (24h) administration of EPA was sufficient to significantly alter the fatty acid composition of WAT via a six fold increase in the percentage of EPA present. This was accompanied by a ten fold increase in serum EPA over the same time period. In addition, the fatty acid composition of murine skeletal muscle demonstrated an eleven fold increase in EPA concentration after 48h, an occurrence which has been previously associated with improved insulin action (Storlien *et al*, 1996). Insulin resistance has been recognised as one of the principal alterations in

carbohydrate metabolism occurring during cachexia (Waterhouse and Kemperman, 1971; Holroyde and Reichard, 1981), hence an improvement in insulin action upon skeletal muscle cells following dietary supplementation with n-3 PUFA may play a role in the stabilisation of skeletal muscle mass observed in EPA supplemented cachectic cancer patients (Wigmore *et al*, 1996a; Wigmore *et al*, 1996b).

The addition of EPA, or other PUFAs, to cultured human cells *in vitro* has been demonstrated to induce alterations in cellular membrane fluidity (Lu *et al*, 1995), cell cycle arrest and apoptosis (Lai *et al*, 1996), to inhibit tumour-induced lipolysis (Tisdale and Beck, 1991) and to modulate signal transduction pathways (Sumida *et al*, 1993). The incorporation of exogenous EPA or DHA into isolated white adipocyte plasma membranes was verified. Hence, any inhibition of tumour-induced lipolysis or AC activity occurring upon exposure of adipocytes to PUFAs may be assumed to be the direct consequence of either an alteration in membrane fluidity, or the modulation of one or more of the stages involved in the signal transduction cascade associated with lipid mobilisation. For example, the β -adrenergic system allied with the beating function of the cardiomyocyte has been demonstrated to be enhanced in cells treated with EPA or DHA (Grynberg *et al*, 1996). Furthermore, DHA has been postulated to influence the formation of metarhodopsin II, the G-protein-activating photointermediate of rhodopsin, a crucial step in visual transduction (Litman and Mitchell, 1996), whilst EPA has been found to covalently modify platelet proteins (Muszbek and Laposata, 1993) and hence may be implicated in the regulation of G-protein α sub-unit functioning (Hallak *et al*, 1994).

It may be postulated, therefore, that the alterations in plasma fatty acid composition manifested upon oral administration of EPA to weight-losing pancreatic cancer patients, may reflect similar alterations in adipose tissue and skeletal muscle fatty acids, as observed in EPA-dosed mice. If such alterations do indeed occur in man, they may be responsible, at least in part, for the stabilisation of subcutaneous adipose tissue and skeletal muscle recorded in cancer patients receiving such dietary supplementation. The mechanisms

underlying such an hypothesis require full investigation. However, the modulatory effects of PUFAs upon the pathways associated with the catabolic actions of tumour-derived factors may be predominantly responsible.

5.4 Results

5.4.1 The Effects of Fatty Acids upon LMF-induced Lipolysis and Adenylate Cyclase Activity in Murine White Adipocytes

Pre-incubation of isolated murine white adipocytes with increasing concentrations of EPA (20:5, n-3) (Figure 5.4.1.1) revealed that whilst lower concentrations (33.1 μ M - 165 μ M) potentiated both isoprenaline- and LMF-stimulated lipolysis, significant inhibition of lipid-mobilisation occurred when higher concentrations (331 μ M) were utilised. Likewise, a significant attenuation of lipolysis was observed when adipocytes were pre-treated with either 331 μ M DHA (22:6, n-3) (Figure 5.4.1.2), or 331 μ M AA (20:4, n-6) (Figure 5.4.1.3), whilst concentrations below 331 μ M were stimulatory.

A comparison of the relative abilities of EPA, DHA and AA to attenuate lipolysis induced by either isoprenaline or LMF was made (Figure 5.4.1.4). This study revealed a less profound inhibition of catabolism in adipocytes pre-treated with DHA when compared to the effects of EPA or AA. In addition, whilst the degree of inhibition produced by EPA and AA could be seen to increase with increasing concentrations of LMF, implying a correlation between the efficacy of the fatty acids and the extent of lipolytic stimulus, the inhibition of lipolysis by DHA did not demonstrate such a relationship.

Equally, pre-incubation of isolated white adipocyte plasma membranes with EPA induced a dose-dependent inhibition of the stimulatory effects of both isoprenaline and LMF (Figure 5.4.1.5). This effect was noted with significantly lower concentrations of EPA than those required to attenuate the stimulation of lipolysis in whole adipocytes, implying an optimisation of the actions of isoprenaline and LMF in this assay. As noted in the lipolysis assay, low concentrations of EPA (25 μ M) potentiated the actions of isoprenaline and LMF upon AC activity, whilst higher concentrations (50 μ M and 100 μ M) demonstrated inhibitory properties.

The Effect of Eicosapentaenoic Acid upon Isoprenaline- and LMF-induced Lipolysis in Murine White Adipocytes

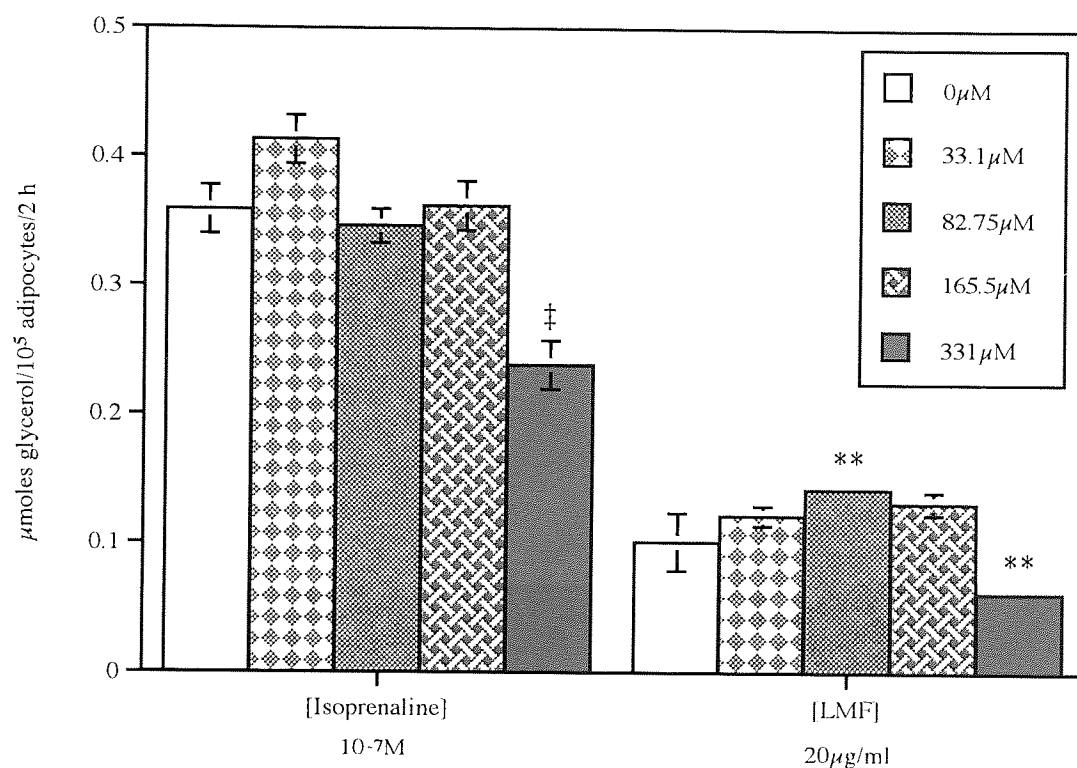


Figure 5.4.1.1 Murine white adipocytes were isolated as described in Section 3.2.4.1. EPA (99% pure) was dissolved in ethanol at a concentration of 10mg/ml and the appropriate concentration added to each 1ml aliquot of fat cells (1×10^5 cells/ml). Ethanol alone was added to control samples and samples pre-incubated at 37°C for 15min prior to the addition of the lipolytic stimulus. Adipocytes were then incubated for a further 2h and glycerol release determined (Section 3.2.4.3). Results expressed as mean \pm SEM, where $n=3$. Statistical analysis performed using Student's t Test, where ** $p \leq 0.05$, † $p \leq 0.001$ compared to 0μM EPA.

The Effect of Docosahexaenoic Acid upon Isoprenaline- and LMF-induced Lipolysis in Murine White Adipocytes

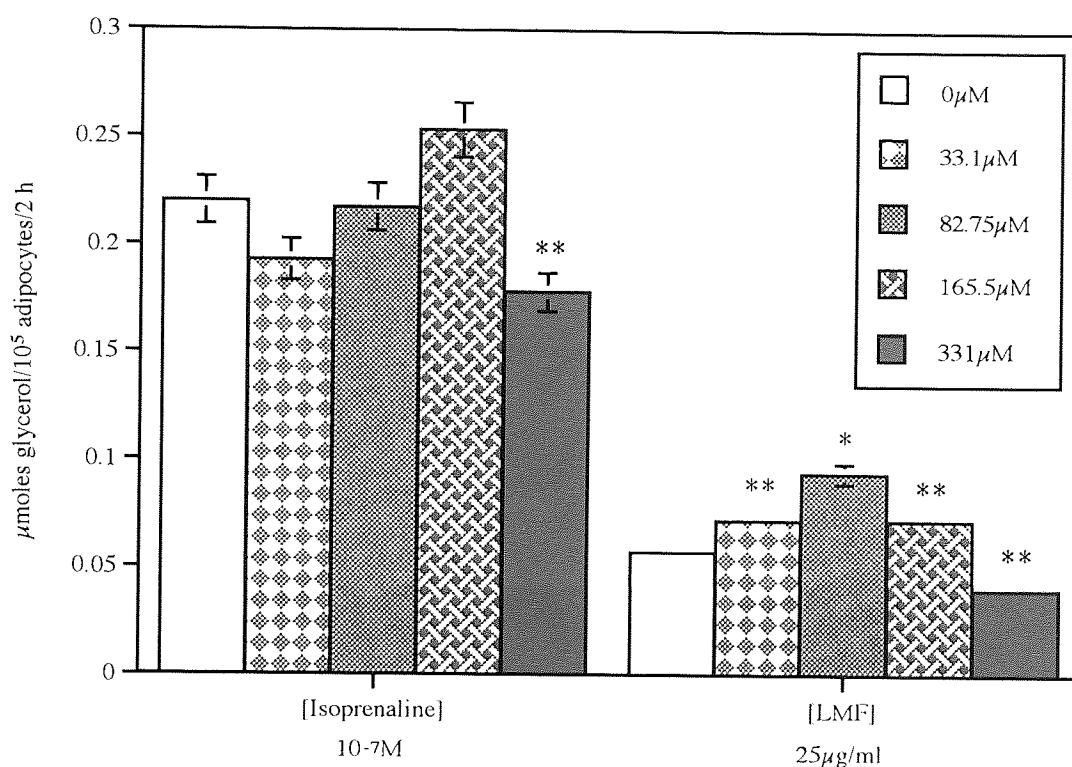


Figure 5.4.1.2 Murine white adipocytes were isolated as described in Section 3.2.4.1. DHA (99% pure) was dissolved in ethanol at a concentration of 10mg/ml and the appropriate concentration added to each 1ml aliquot of fat cells (1×10^5 cells/ml). Ethanol alone was added to control samples and samples pre-incubated at 37°C for 15min prior to the addition of the lipolytic stimulus. Adipocytes were then incubated for a further 2h and glycerol release determined (Section 3.2.4.3). Results expressed as mean \pm SEM, where $n=3$. Statistical analysis performed using Student's t Test, where ** $p \leq 0.05$, * $p \leq 0.01$ compared to 0μM DHA.

The Effect of Arachidonic Acid upon Isoprenaline- and LMF-induced Lipolysis in Murine White Adipocytes

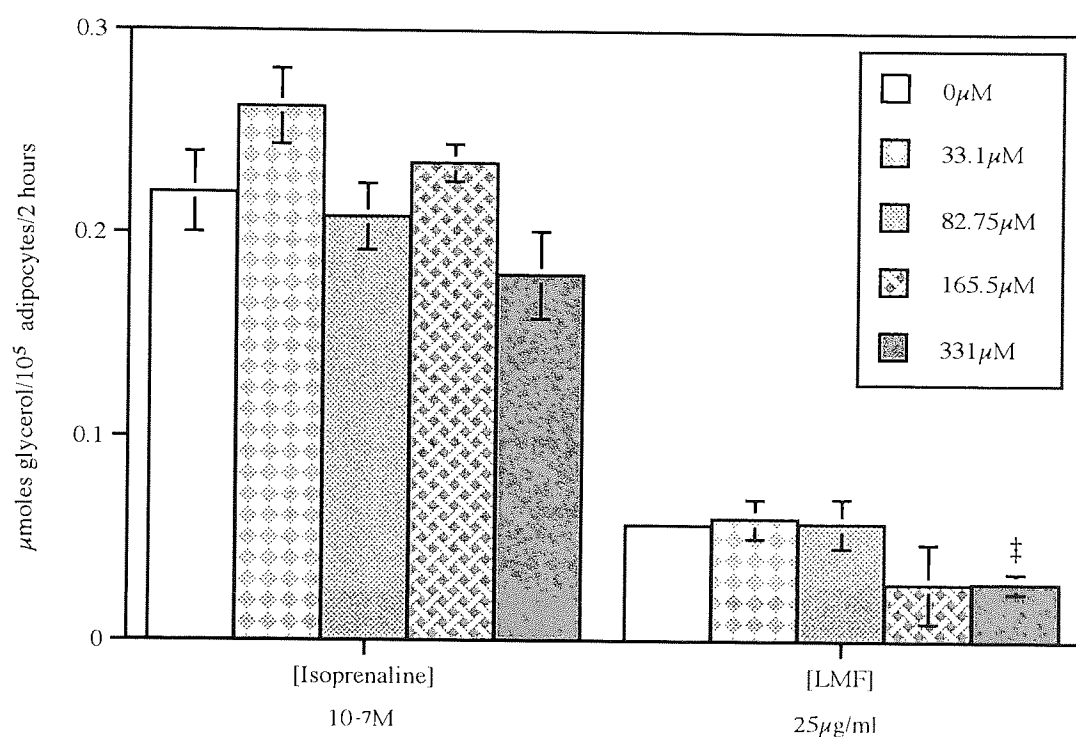


Figure 5.4.1.3 Murine white adipocytes were isolated as described in Section 3.2.4.1. AA (99% pure) was dissolved in ethanol at a concentration of 10mg/ml and the appropriate concentration added to each 1ml aliquot of fat cells (1×10^5 cells/ml). Ethanol alone was added to control samples and samples pre-incubated at 37°C for 15min prior to the addition of the lipolytic stimulus. Adipocytes were then incubated for a further 2h and glycerol release determined (Section 3.2.4.3). Results expressed as mean \pm SEM, where $n=3$. Statistical analysis performed using Student's t Test, where $\ddagger p \leq 0.001$ compared to 0μM AA.

The Comparative Effects of EPA, DHA and AA upon Isoprenaline- and LMF-induced Lipolysis in Murine White Adipocytes

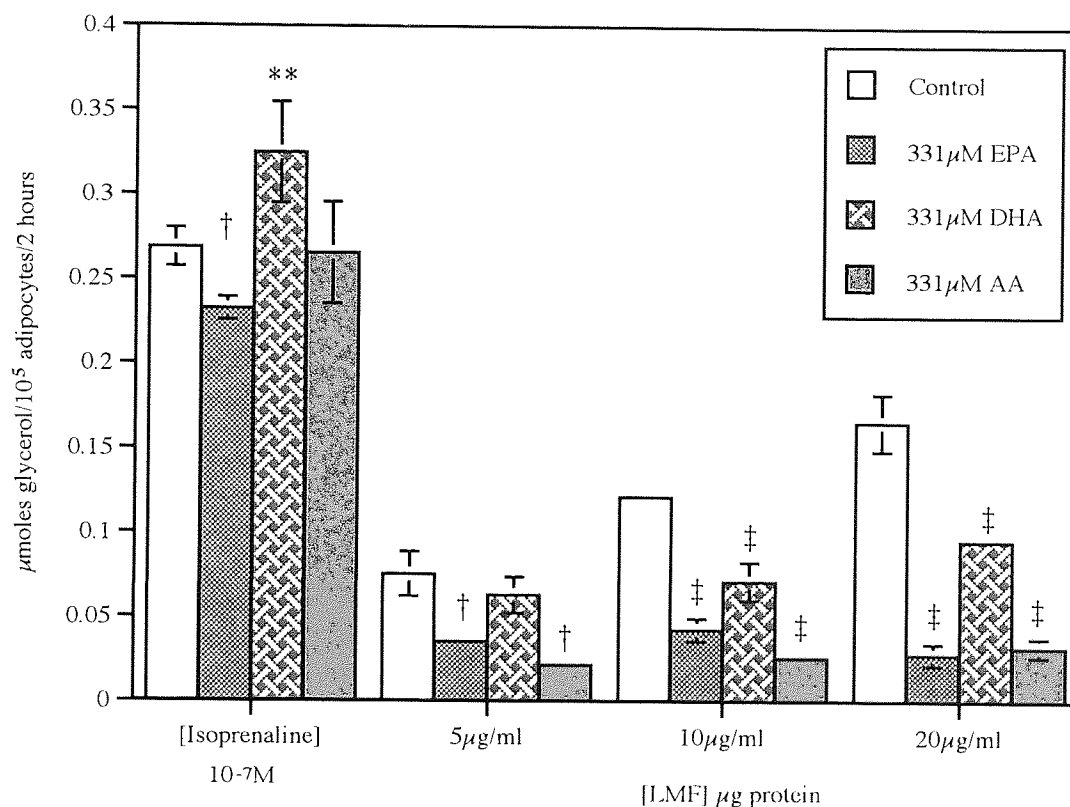


Figure 5.4.1.4 Murine white adipocytes were isolated as described in Section 3.2.4.1. EPA, DHA and AA (all 99% pure) were dissolved in ethanol at a concentration of 10mg/ml, and 331 μM of each added to the appropriate samples (1x10⁵ adipocytes/ml). Ethanol alone was added to control samples, and samples pre-incubated at 37°C for 15min prior to the addition of the lipolytic stimulus. Adipocytes were then incubated for a further 2h and glycerol release determined (Section 3.2.4.3). Results expressed as mean ± SEM, where n=3. Statistical analysis performed using Student's t Test, where **p≤0.05, †p≤0.005, ‡p≤0.001 compared to 0 μM fatty acid.

Effects of Eicosapentaenoic Acid upon Adenylate Cyclase Activity

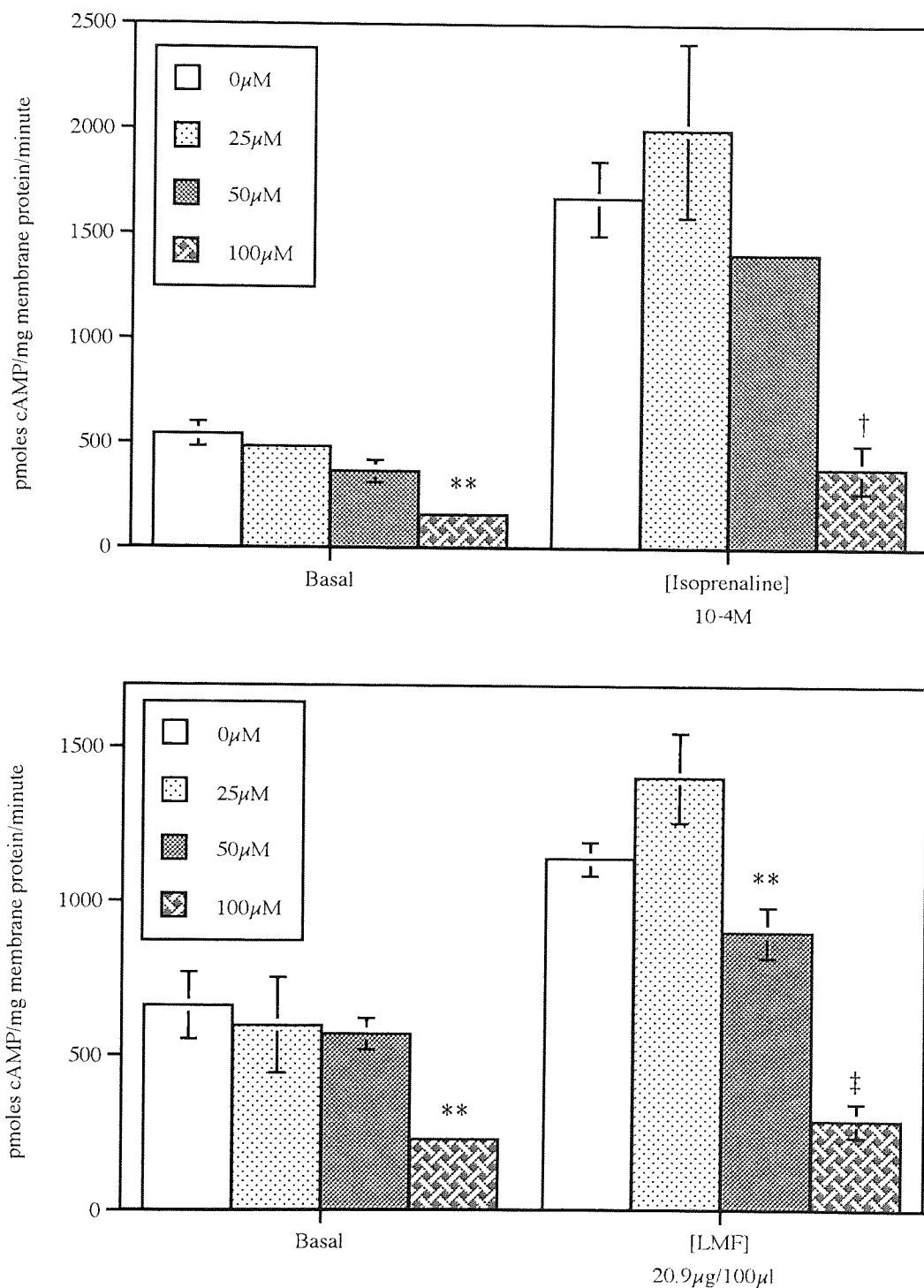


Figure 5.4.1.5 Adipocyte membranes (30 μg) were pre-treated (30min at room temperature) with 99% pure EPA dissolved in ethanol (10mg/ml) at either 25 μM, 50 μM or 100 μM prior to addition to the incubation mixture (Section 3.5.2). Results expressed as mean ± SEM where n=3. Statistical analysis performed using Student's t Test, **p ≤ 0.05, †p ≤ 0.005, ‡p ≤ 0.001 compared to 0 μM.

Effect of Docosahexaenoic Acid upon Adenylate Cyclase Activity

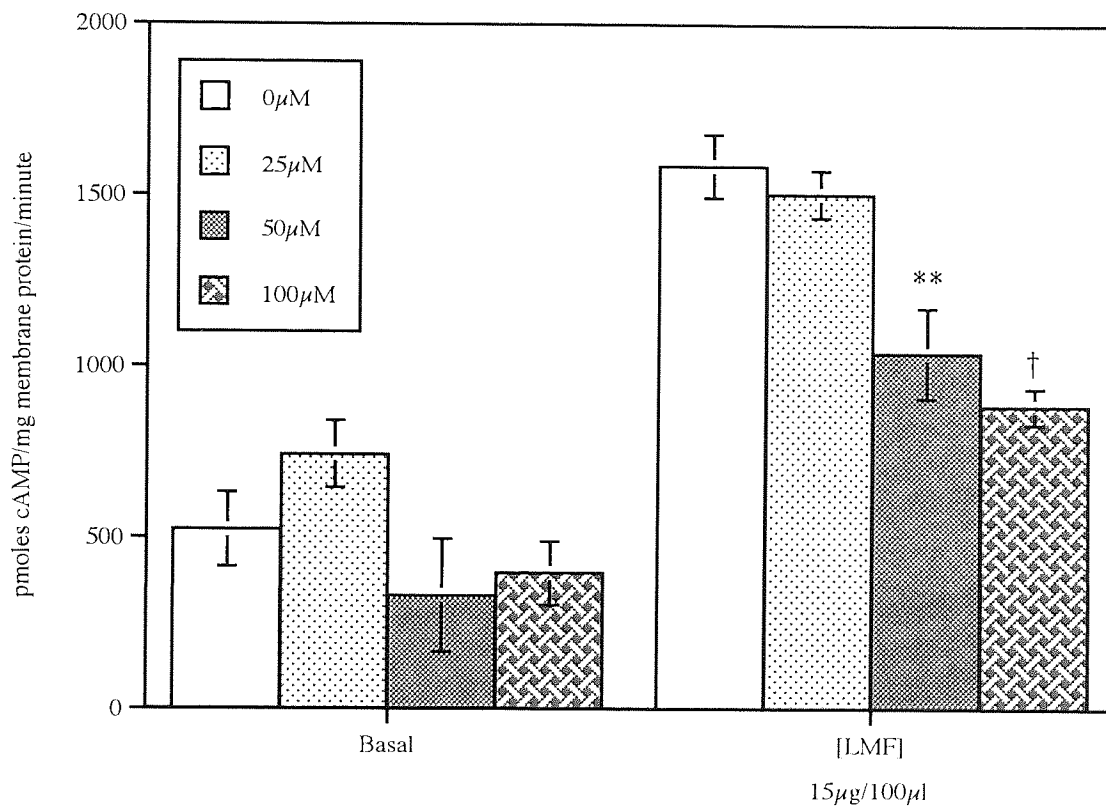


Figure 5.4.1.6 Adipocyte plasma membranes were isolated as described in Section 3.5.1. DHA (99% pure) was dissolved in ethanol (10mg/ml) and the appropriate concentration added to each membrane sample (27µg). Membranes were pre-incubated for 30min at room temperature prior to addition to the incubation mixture (Section 3.5.2). Results expressed as mean \pm SEM, where n=3. Statistical analysis performed using Student's t Test, where **p<0.05, †p<0.005 compared to 0µM DHA.

Comparative Effects of EPA and DHA upon Adenylate Cyclase Activity

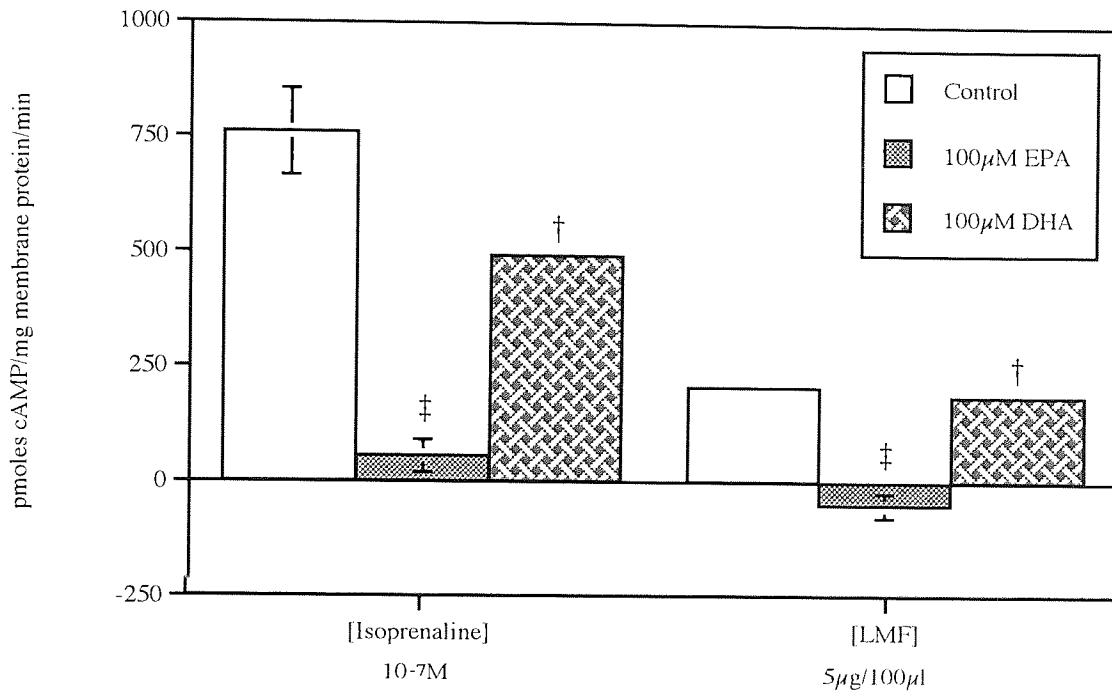


Figure 5.4.1.7 Adipocyte plasma membranes were isolated as described in Section 3.5.1. EPA and DHA (both 99% pure) were dissolved in ethanol (10mg/ml) and the appropriate concentration added to each membrane sample (25µg). Membranes were pre-incubated for 30min at room temperature prior to addition to the incubation mixture (Section 3.5.2). Results expressed as mean \pm SEM, where n=3. Statistical analysis performed using Student's t Test, where †p<0.005, ‡p<0.001 compared to 0µM fatty acid.

Effects of Arachidonic Acid upon Adenylate Cyclase Activity

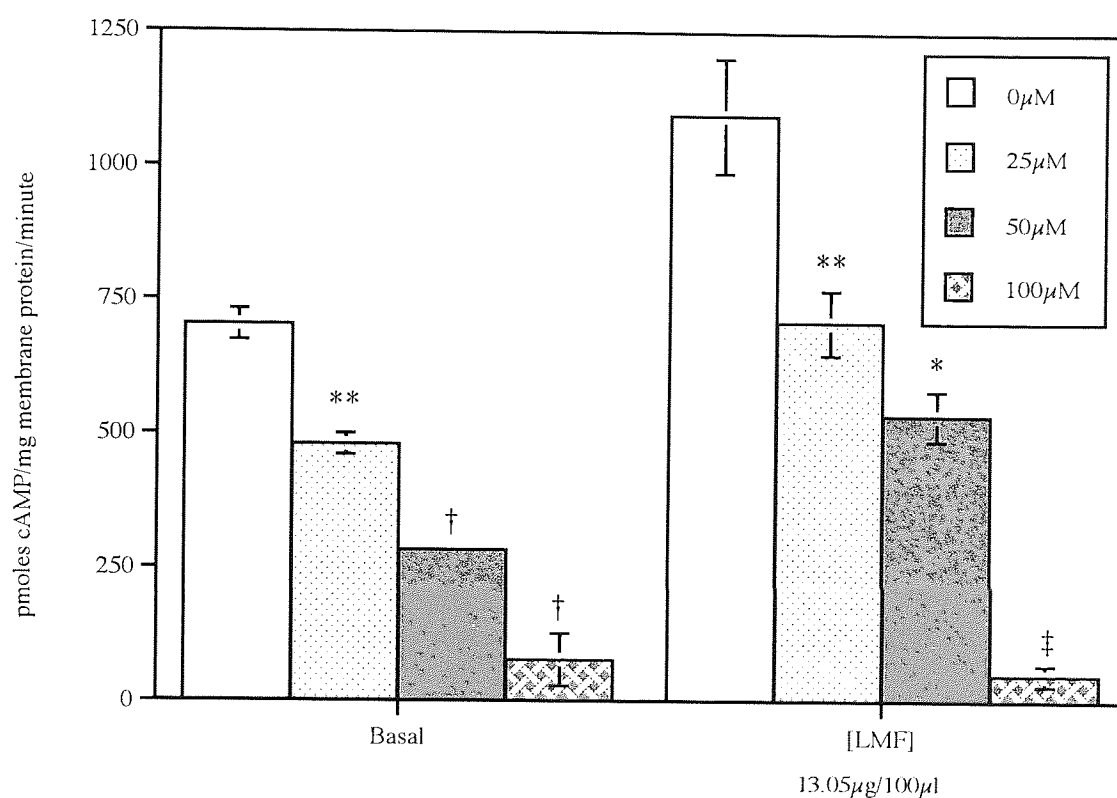


Figure 5.4.1.8 Adipocyte plasma membranes were isolated as described in Section 3.5.1. AA (99% pure) was dissolved in ethanol (10mg/ml) and the appropriate concentration added to each membrane sample (26µg). Membranes were pre-incubated for 30min at room temperature prior to addition to the incubation mixture (Section 3.5.2). Results expressed as mean \pm SEM, where $n=3$. Statistical analysis performed using Student's t Test, where ** $p \leq 0.05$, * $p \leq 0.01$, † $p \leq 0.005$, ‡ $p \leq 0.001$ compared to 0µM AA.

Similarly, 50 μ M and 100 μ M DHA significantly inhibited the stimulation of AC activity by LMF (Figure 5.4.1.6), however as noted previously, this effect was not as profound as that demonstrated by EPA (Figure 5.4.1.7). Pre-incubation of adipocyte plasma membranes with AA also dose-dependently attenuated the actions of LMF (Figure 5.4.1.8), with all concentrations of AA utilised proving inhibitory.

5.4.2 The Effects of Oral Administration of Eicosapentaenoic Acid upon Adenylate Cyclase Activity in White Adipocyte Plasma Membranes and Tumour Production of LMF

White adipocyte plasma membranes prepared from mice which had received oral EPA (1.25g/kg) demonstrated an attenuated response to forskolin (Figure 5.4.2.1), implying a direct interaction between EPA and AC. In addition, an attenuation of the responsiveness of the membranes to both isoprenaline and LMF could be observed (Figure 5.4.2.1). Similarly, whilst adipocyte membranes isolated from cachectic MAC16 tumour-bearing mice demonstrated a significantly elevated response to both isoprenaline and LMF, post oral administration of a single dose of EPA (1.25g/kg) significantly attenuated this enhanced sensitivity, returning membrane responsiveness to that observed using membranes from non tumour-bearing mice (Figure 5.4.2.2).

Given the short term (24h) effects of EPA upon isoprenaline and LMF-induced AC activity in membranes prepared from both cachectic and non-cachectic mice, the effects of a single dose of 1.25g/kg EPA upon tumour production of LMF was investigated. Figure 5.4.2.3 illustrates that there were no significant differences in the induction of lipolysis in murine white adipocytes by LMF purified from EPA dosed mice when compared to that from untreated animals. Hence the ability of EPA to attenuate the progression of cachexia in mice bearing the MAC16 tumour model appears to be a consequence of the inhibition of the signal transduction pathway associated with lipid catabolism, rather than the result of any down-regulation of the production of LMF by the tumour itself.

Effect of Oral Administration of EPA upon the Stimulation of Adenylate Cyclase Activity by Isoprenaline, Forskolin and LMF

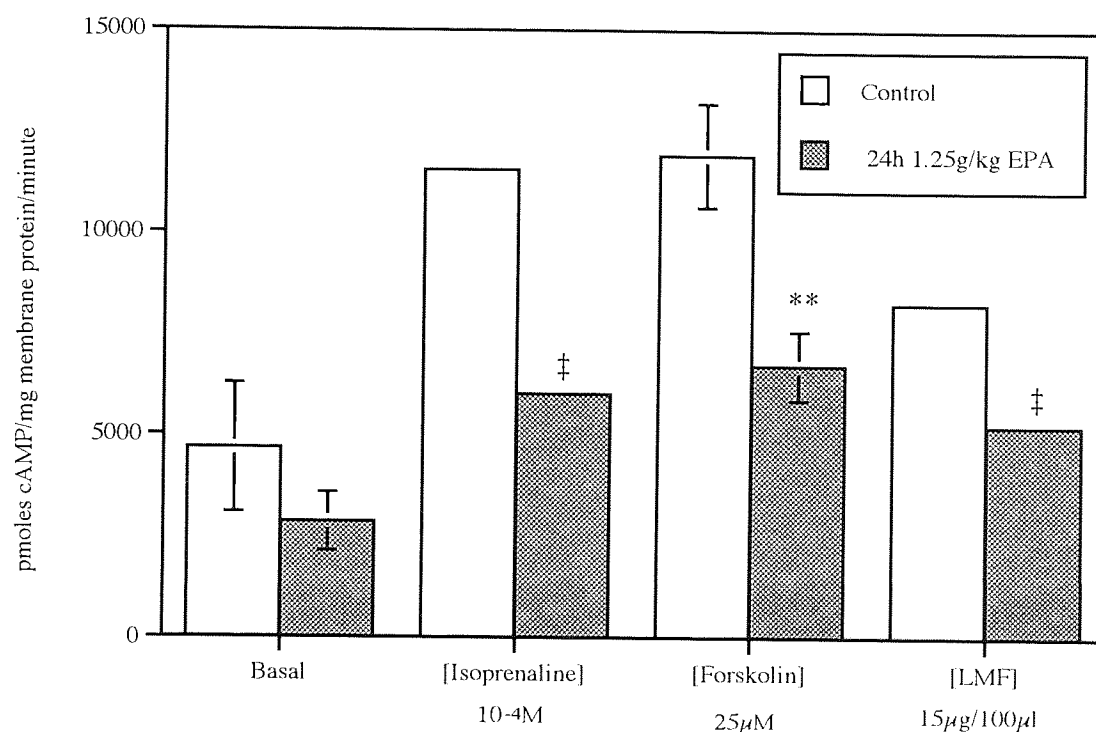


Figure 5.4.2.1 A single dose of EPA (1.25g/kg) was administered to male BKW mice. After 24h mice were killed by cervical dislocation, their epididymal fat pads removed and adipocyte plasma membranes prepared together with membranes from undosed animals (Section 3.5.1). Membranes (both 28 μg/sample) were added to the incubation mixture, and AC activity, in response to isoprenaline, forskolin and LMF, measured as previously described (Section 3.5.2). Results expressed as mean ± SEM, where n=3. Statistical analysis performed using Student's t Test, where **p≤0.05, ‡p≤0.001, compared to control.

Effect of Oral Administration of EPA upon the Stimulation of Adenylate Cyclase Activity in Adipocyte Membranes Isolated from Cachectic Mice

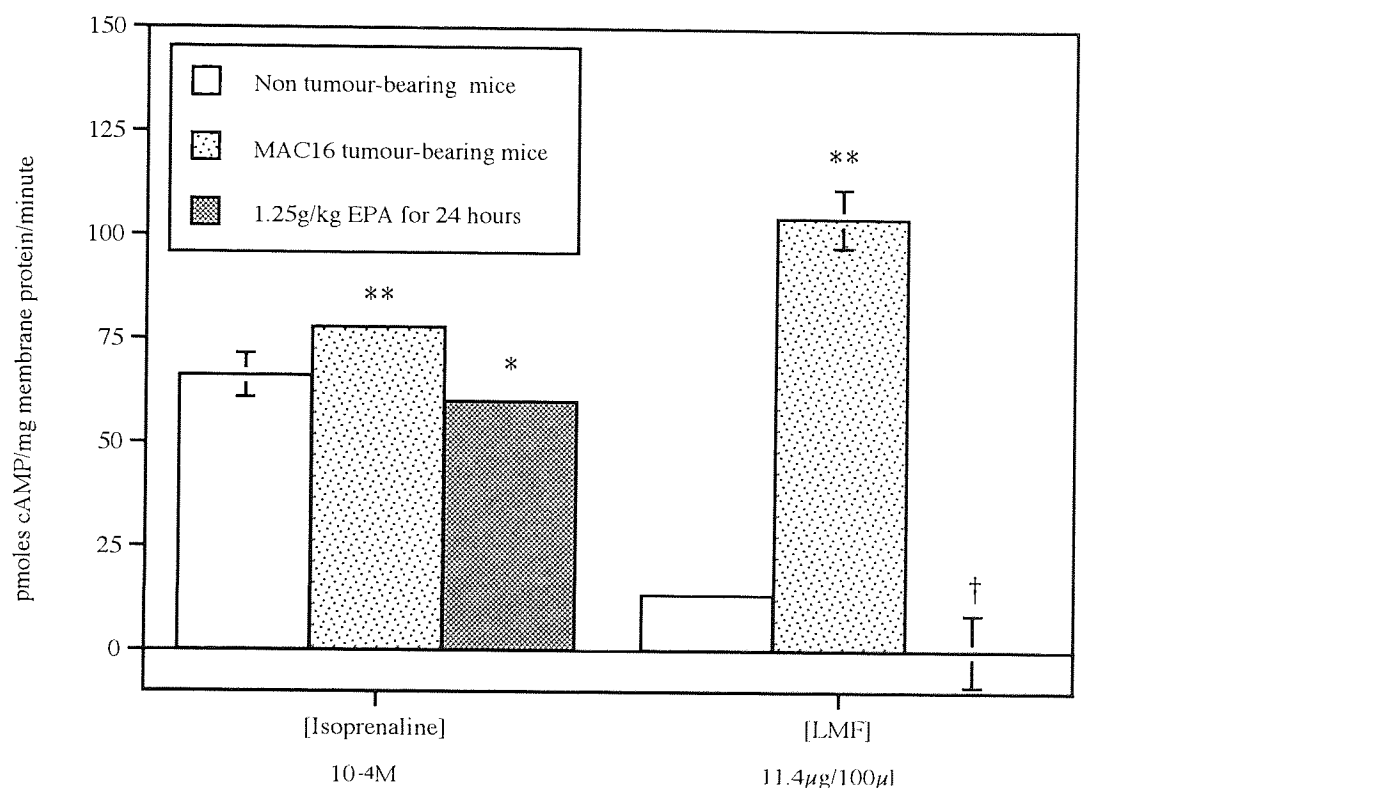


Figure 5.4.2.2 A single dose of EPA (1.25g/kg) was administered to cachectic MAC16 tumour-bearing, male NMRI mice. After 24h mice were killed by cervical dislocation, their epididymal fat pads removed and adipocyte plasma membranes prepared together with membranes from non tumour-bearing NMRI mice and undosed MAC16 tumour-bearers (Section 3.5.1). Membranes (all 35 μg/sample) were added to the incubation mixture, and AC activity, in response to isoprenaline and LMF, measured as previously described (Section 3.5.2). Results expressed as mean ± SEM, where n=3. Statistical analysis performed using Student's t Test, where **p≤0.05 compared to control, and *p≤0.01, †p≤0.005, compared to undosed MAC16 tumour-bearing mice.

Effect of Oral Administration of Eicosapentaenoic Acid on the Production of LMF by the MAC16 Tumour

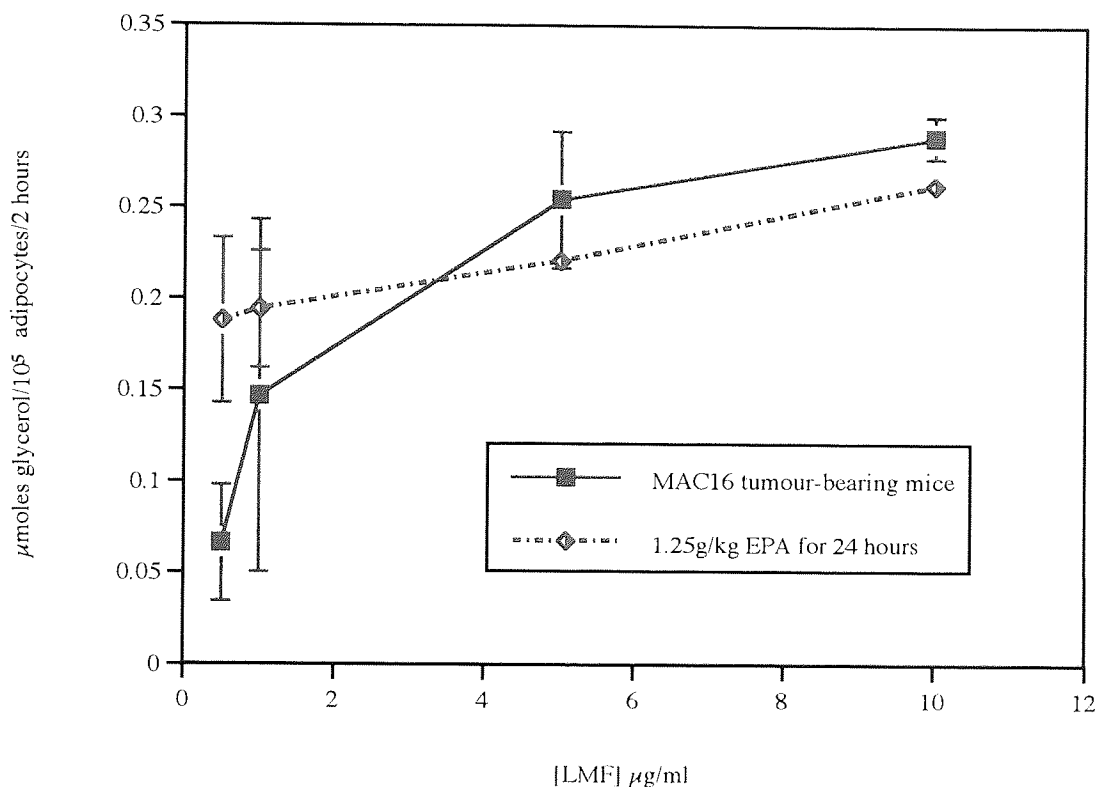


Figure 5.4.2.3 A single dose of EPA (1.25g/kg) was administered to cachectic MAC16 tumour-bearing mice. After 24h mice were killed by cervical dislocation, their tumours excised and LMF purified as described in Section 3.2.3. Isolated white murine adipocytes (1×10^5 cells/ml) were incubated with known concentrations of LMF for 2h, and glycerol release subsequently determined (Section 3.2.4). Results expressed as mean \pm SEM, where $n=3$.

5.4.3 The Effects of Pertussis Toxin upon the Inhibition of Lipolysis and Adenylate Cyclase Activity in Murine White Adipocytes by Fatty Acids

Treatment of isolated white murine adipocytes with pre-activated pertussis toxin, prior to the addition of EPA, markedly inhibited the attenuation of the effects of isoprenaline and LMF associated with EPA pre-treatment (Figure 5.4.3.1). Hence adipocytes exposed to pertussis toxin and EPA exhibited an enhanced response to isoprenaline and LMF, indicating the induction of alterations within the adipocyte by pertussis toxin which prevented the inhibitory actions of the PUFA. Furthermore, pre-treatment of isolated murine white adipocyte plasma membrane fractions with pre-activated pertussis toxin reversed the inhibitory actions of EPA upon both isoprenaline-, and LMF-stimulated AC activity (Figure 5.4.3.2), indicating that EPA exerts at least part of its inhibitory actions via an interaction with some component of the cell plasma membrane, namely an inhibitory G-protein α sub-unit ($G\alpha_i$).

Conversely, pertussis toxin pre-treatment was unable to inhibit the effect of DHA upon isoprenaline-, and LMF-induced lipolysis (Figure 5.4.3.3) indicating that DHA does not rely solely upon an interaction with $G\alpha_i$ to exert its inhibitory actions. Hence, adipocytes pre-treated with pre-activated pertussis toxin nevertheless demonstrated an attenuated response to lipolytic stimuli upon exposure to DHA. It is likely, therefore, that the inhibitory mechanisms of these two PUFAs are disparate.

Interestingly, whilst pertussis toxin reversed the inhibition of LMF-induced lipolysis by AA, it had no significant effect upon the attenuation of isoprenaline-stimulated lipolysis by AA (Figure 5.4.3.4). Such a finding would appear to imply that AA inhibits the induction of lipolysis by isoprenaline via a direct interaction with AC, and conversely, it is an interaction with $G\alpha_i$ which enables AA to attenuate the effects of LMF. It is possible that this phenomenon is indicative of the roles of two different receptors in the induction of lipolysis by isoprenaline and LMF.

Effect of Pertussis Toxin on the Inhibition of Isoprenaline- and LMF-induced Lipolysis by Eicosapentaenoic Acid

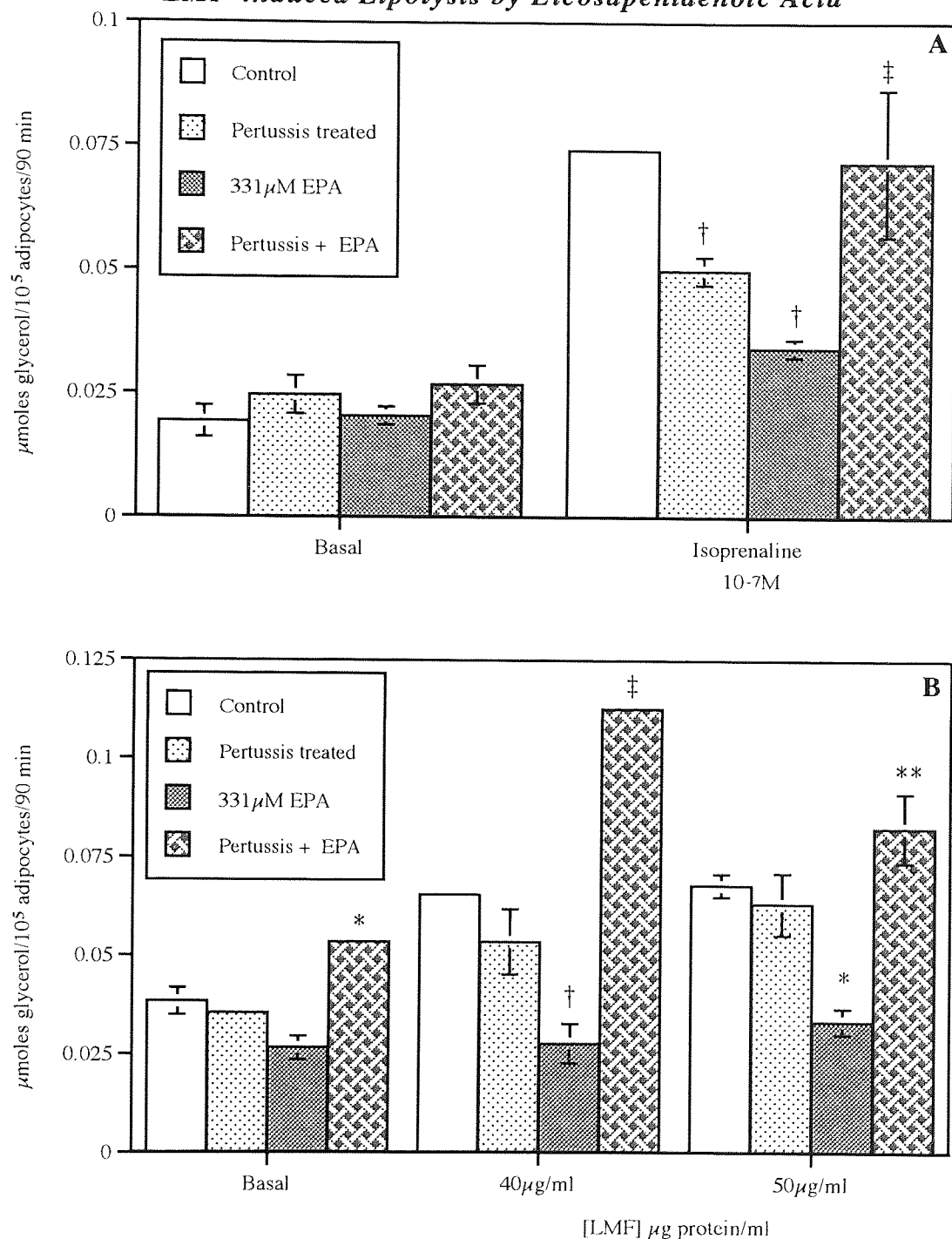


Figure 5.4.3.1 Adipocytes were treated with pre-activated pertussis toxin as described (Section 3.6.2). Isoprenaline (A) or LMF (B) was added to each 1ml aliquot of cells (1×10^5 cells/ml) and adipocytes incubated for a further 90min. Glycerol release was subsequently determined (Section 3.2.4.3). Results expressed as mean \pm SEM where $n=3$. Statistical analysis performed using Student's t Test, where $\dagger p \leq 0.005$, pertussis treated compared to control; $* p \leq 0.01$, $\dagger p \leq 0.005$ 331 μ M EPA compared to control, and $** p \leq 0.05$, $* p \leq 0.01$, $\ddagger p \leq 0.001$ Pertussis + 331 μ M EPA compared to 331 μ M EPA.

Effect of Pertussis Toxin on the Inhibition of Isoprenaline- and LMF-Stimulated Adenylate Cyclase Activity by Eicosapentaenoic Acid

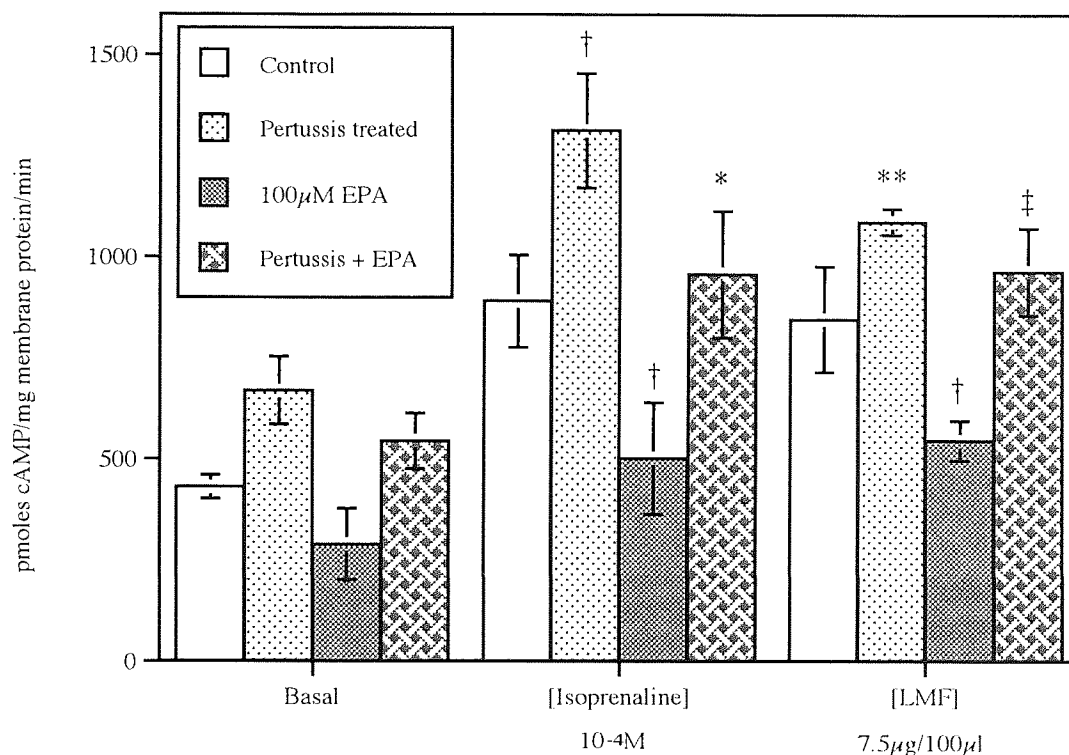


Figure 5.4.3.2 Adipocyte plasma membranes (typically 30 µg) were pre-treated with pre-activated pertussis toxin prior to the addition of EPA (100 µM) (Section 3.6.3). membranes were subsequently added to the incubation mixture and the stimulation of AC by isoprenaline and LMF determined (Section 3.5.2). Results expressed as mean ± SEM, where n=4. Statistical analysis performed using Student's t Test where: **p ≤ 0.05, †p ≤ 0.005, pertussis treated from control; †p ≤ 0.005, 100 µM EPA from control and *p ≤ 0.01, ‡p ≤ 0.001 pertussis + 100 µM EPA from 100 µM EPA.

Effect of Pertussis Toxin on the Inhibition of Isoprenaline- and LMF-Stimulated Lipolysis by Docosahexaenoic Acid

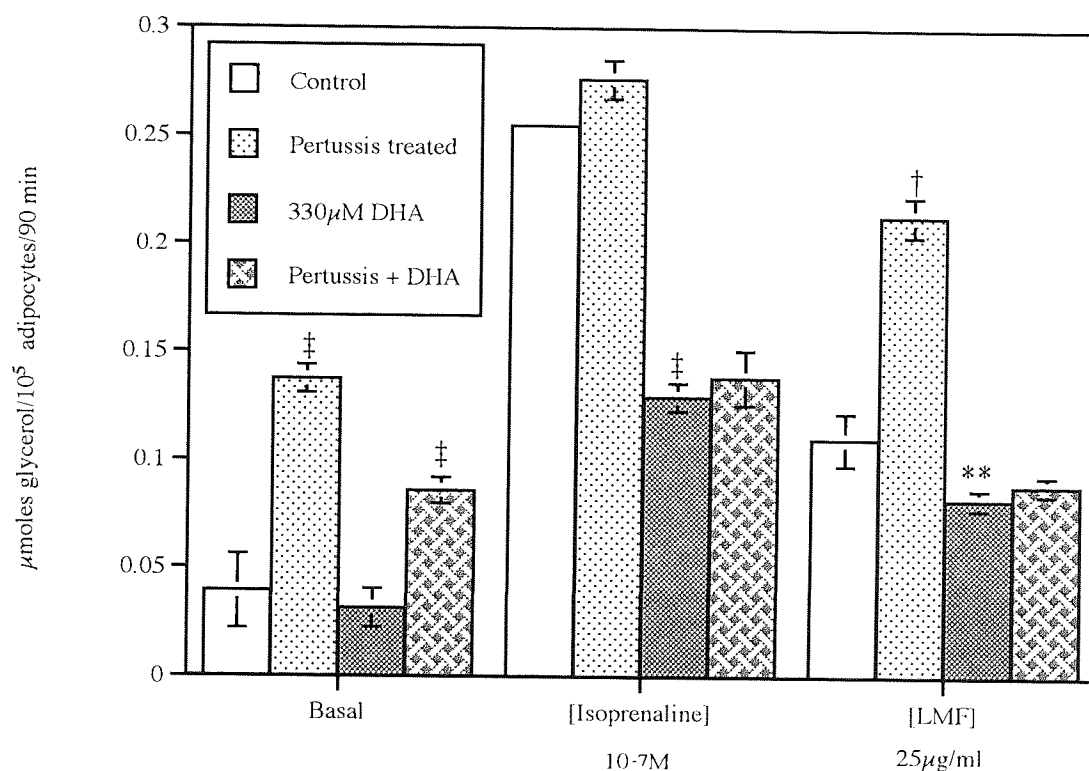


Figure 5.4.3.3 Murine white adipocytes (1×10^5 cells/ml) were pre-treated with pre-activated pertussis toxin prior to the addition of DHA ($331 \mu\text{M}$) (Section 3.6.2). Isoprenaline and LMF were subsequently added to the incubation mixture and the stimulation of lipolysis determined (Section 3.2.4). Results expressed as mean \pm SEM, where $n=3$. Statistical analysis performed using Student's t Test where: † $p \leq 0.005$, ‡ $p \leq 0.001$ pertussis treated from control; ** $p \leq 0.05$, ‡ $p \leq 0.001$, $331 \mu\text{M}$ DHA from control.

Effect of Pertussis Toxin on the Inhibition of Isoprenaline- and LMF-Stimulated Lipolysis by Arachidonic Acid

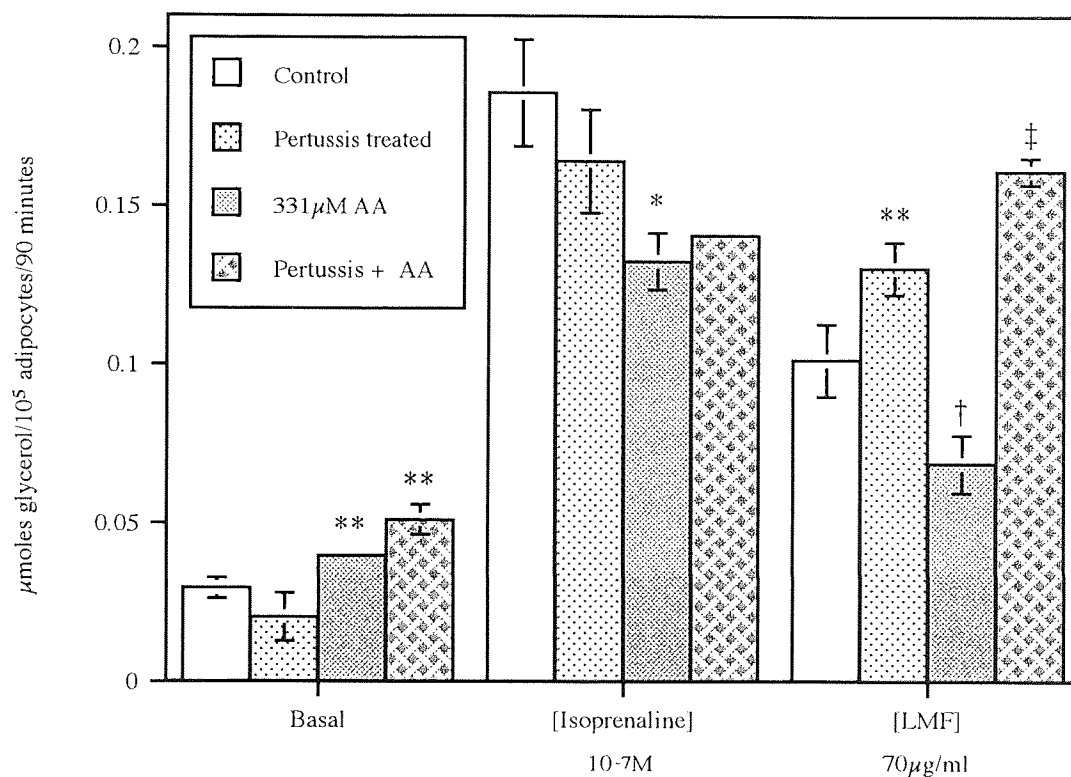


Figure 5.4.3.4 Murine white adipocytes (1×10^5 cells/ml) were pre-treated with pre-activated pertussis toxin prior to the addition of AA ($331 \mu\text{M}$) (Section 3.6.2). Isoprenaline and LMF were subsequently added to the incubation mixture and the stimulation of lipolysis determined (Section 3.2.4). Results expressed as mean \pm SEM, where $n=3$. Statistical analysis performed using Student's t Test where: $\dagger p \leq 0.005$, $\ddagger p \leq 0.001$ pertussis treated from control; $** p \leq 0.05$, $\ddagger p \leq 0.001$, $331 \mu\text{M}$ AA from control.

5.4.4 Effect of Oral Administration of Eicosapentaenoic Acid upon Circulatory p24 Levels in Advanced Pancreatic Cancer Patients

It is possible that oral administration of EPA, in the form of MaxEPA capsules, to advanced weight-losing pancreatic cancer patients could attenuate the progression of cachexia via a down-regulation of tumour-derived factor production. Western blotting of patient plasma (Figure 5.4.4.1B) demonstrated an apparent decrease in immunoreactive band density with EPA dietary supplementation. However, comparison of plasma samples prepared from cachectic cancer patients with samples from normal controls revealed a non-specific interaction of the MAb with some plasma component, perhaps immunoglobulins, present in all subjects (Figure 5.4.4.2).

Attempts to isolate p24 from other plasma components by immunoprecipitation were unsuccessful, with an even greater degree of non-specific interaction occurring (Figure 5.4.4.3). It is likely, therefore, that antibodies present within the plasma samples were reacting non-specifically during immunodetection, possibly with the HRP-conjugated protein A solution employed to detect MAb binding. The 55kDa Western positive band detected in these blots is probably representative of non-specific MAb binding to immunoglobulin heavy chain. Until a more efficacious method to separate immunoglobulins present in plasma from other plasma proteins has been developed, it is unlikely that it will be possible to study any alterations in tumour factor production occurring during therapy by Western blotting.

5.4.5 Alterations in Adipocyte Plasma Membrane G-proteins Occurring During the Progression of Cancer Cachexia, and the Effects of Oral Administration of Eicosapentaenoic Acid

Electrophoretic separation, and immunodetection of the $G\alpha_i$ and $G\alpha_s$ sub-units present in the plasma membranes isolated from the white adipocytes of mice transplanted with an experimental cancer cachexia model, the MAC16 colon adenocarcinoma, revealed an alteration of immunoreactivity with the progression of the cachectic state. A comparison of membranes prepared from MAC16 tumour-bearing mice which had not lost weight with

Effect of Dietary Supplementation with MaxEPA Capsules upon Circulatory Levels of p24 in Cachectic Cancer Patients

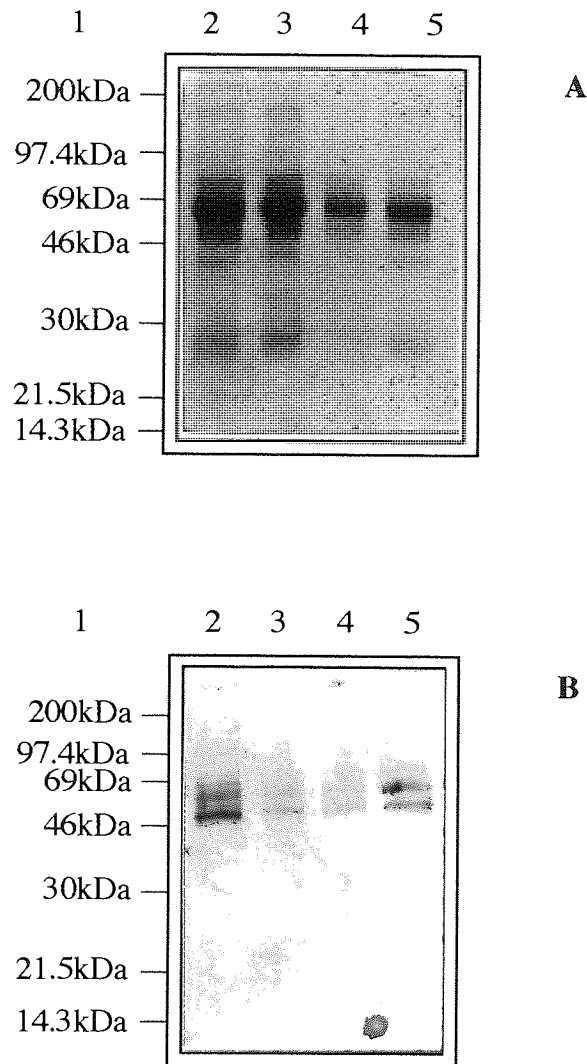


Figure 5.4.4.1 Samples of plasma from a single weight-losing pancreatic cancer patient receiving dietary supplementation with EPA, in the form of MaxEPA capsules, were prepared (Section 3.7.1.1) ($1\mu\text{g}$ each) and separated using a 15% resolving gel. Electrophoresed proteins were either visualised by Coomassie staining (A) (Section 3.7.3) or transferred to nitrocellulose (Section 3.7.4) and immunoreactive bands detected using the mouse MAb (Sections 3.7.4.1.1 and 3.7.4.2) (B). Lane 1 - Molecular weight markers; Lane 2 - patient 1, May 1994; Lane 3 - patient 1, June 1994; Lane 4 - patient 1, August 1994; Lane 5 - patient 1, November 1994.

*Comparison of Circulatory Levels of p24 in Cachectic Cancer Patients
Receiving MaxEPA Capsules with Healthy Control Subjects*

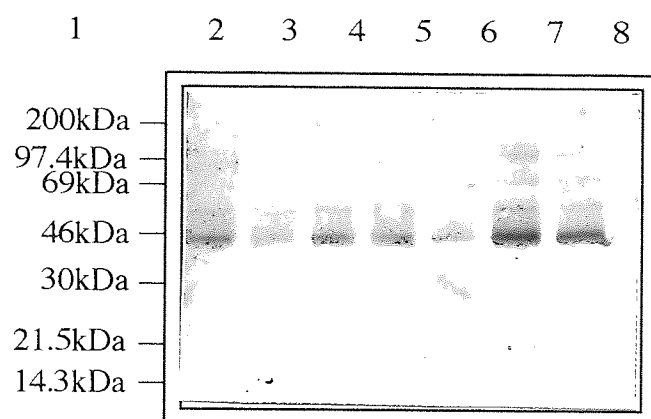


Figure 5.4.4.2 Samples of plasma from weight-losing pancreatic cancer patients receiving dietary supplementation with EPA, in the form of MaxEPA capsules, and from healthy non tumour-bearing controls, were prepared ($1\mu\text{g}$ each) and separated using a 15% resolving gel (Sections 3.7.1.1 and 3.7.2). Electrophoresed proteins were transferred to nitrocellulose (Section 3.7.4) and immunoreactive bands detected using the mouse MAb (Sections 3.7.4.1.1 and 3.7.4.2). Lane 1 - Molecular weight markers; Lane 2 - control plasma; Lane 3 - patient 2, July 1994; Lane 4 - patient 2, August 1994; Lane 5 - patient 2, October 1994; Lane 6 - control plasma; Lane 7 - patient 3, July 1994; Lane 8 - patient 3, August 1994.

*Comparison of Circulatory Levels of p24 in Cachectic Cancer Patients
Receiving MaxEPA Capsules with Healthy Controls Following
Immunoprecipitation*

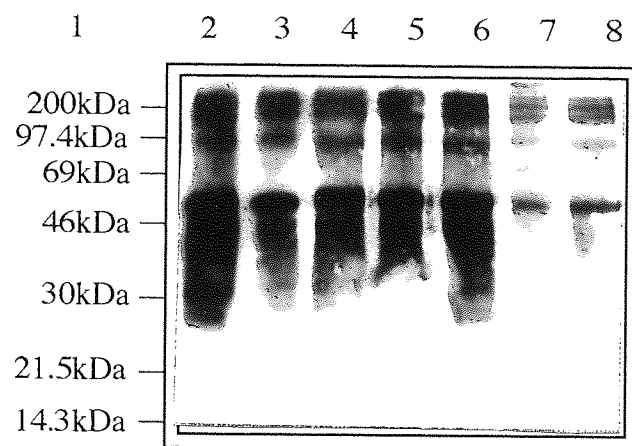


Figure 5.4.4.3 Samples of plasma from weight-losing pancreatic cancer patients receiving dietary supplementation with MaxEPA capsules, and from healthy non tumour-bearing controls, were immunoprecipitated using MAb and protein A sepharose (Section 3.7.1.2) ($1\mu\text{g}$ each), denatured and separated using a 15% resolving gel. Electrophoresed proteins were transferred to nitrocellulose (Section 3.7.4) and immunoreactive bands detected using the mouse MAb (Sections 3.7.4.1.1 and 3.7.4.2). Lane 1 - Molecular weight markers; Lane 2 - control plasma; Lane 3 - patient 1, May 1994; Lane 4 - patient 1, June 1994; Lane 5 - patient 1, August 1994; Lane 6 - patient 1, November 1994; Lane 7 - control plasma; Lane 8 - control plasma.

those from non tumour-bearing mice demonstrated a five fold increased density of the 40kDa band detected by anti-G α_i in non-cachectic tumour-bearing mice (Figure 5.4.5.1). This G α_i band demonstrated a decreased density in tumour-bearing mice with 5% weight loss, and decreased further at 10% weight loss, the quantity of G α_i present in adipocyte membranes thereafter increased with additional host wasting. Conversely, G α_s could be seen to be decreased by approximately 60% in membranes from non-cachectic tumour-bearing mice when compared to non tumour-bearers (Figure 5.4.5.2). The quantity of G α_s detected by anti-G α_s in white adipocyte membranes could be seen to increase as cachexia progressed, being maximal with 10% weight loss.

Hence, it may be observed that there is a concomitant increase in G α_s which mirrors the decreased G α_i in adipocyte plasma membranes isolated from mice bearing a cachexigenic tumour with various degrees of weight loss. The ratio of G α_s to G α_i detected in adipocyte plasma membranes decreased markedly in those mice demonstrating resistance to the cachexigenic actions of the MAC16 tumour. Progression of the cachectic state induced an increase in G α_s :G α_i to approximately three times that noted in adipocytes from non tumour-bearing mice when tumour LMF production was maximal (Table 5.4.5.1). A subsequent decrease in G α_s :G α_i to one third of control values was observed with additional weight loss, perhaps as a consequence of reduced LMF production. EPA attenuated G α_s :G α_i to approximately one quarter of that present in undosed mice with an identical degree of cachexia, suggesting a normalisation of the tumour-induced effect. Such alterations in G-protein expression may occur due to the influence of LMF upon the adipocyte, with high circulatory concentrations of LMF inducing alterations in G-protein expression which facilitate it's lipolytic actions; that is an up-regulation of G α_s and a down-regulation of G α_i . These changes may serve to maximise the lipid-mobilising actions of LMF, hence enhancing the efficacy of adipose tissue fatty acid mobilisation and ensuring an adequate supply for tumour growth and metabolism. The administration of 1.25g/kg 95% pure EPA to MAC16 tumour-bearing mice with 10% weight loss produced an increase in G α_i and a decrease in G α_s detectable by Western blotting.

Immunodetection of Gα_i in White Adipocyte Plasma Membranes Isolated from Cachectic MAC16 Tumour-bearing Mice

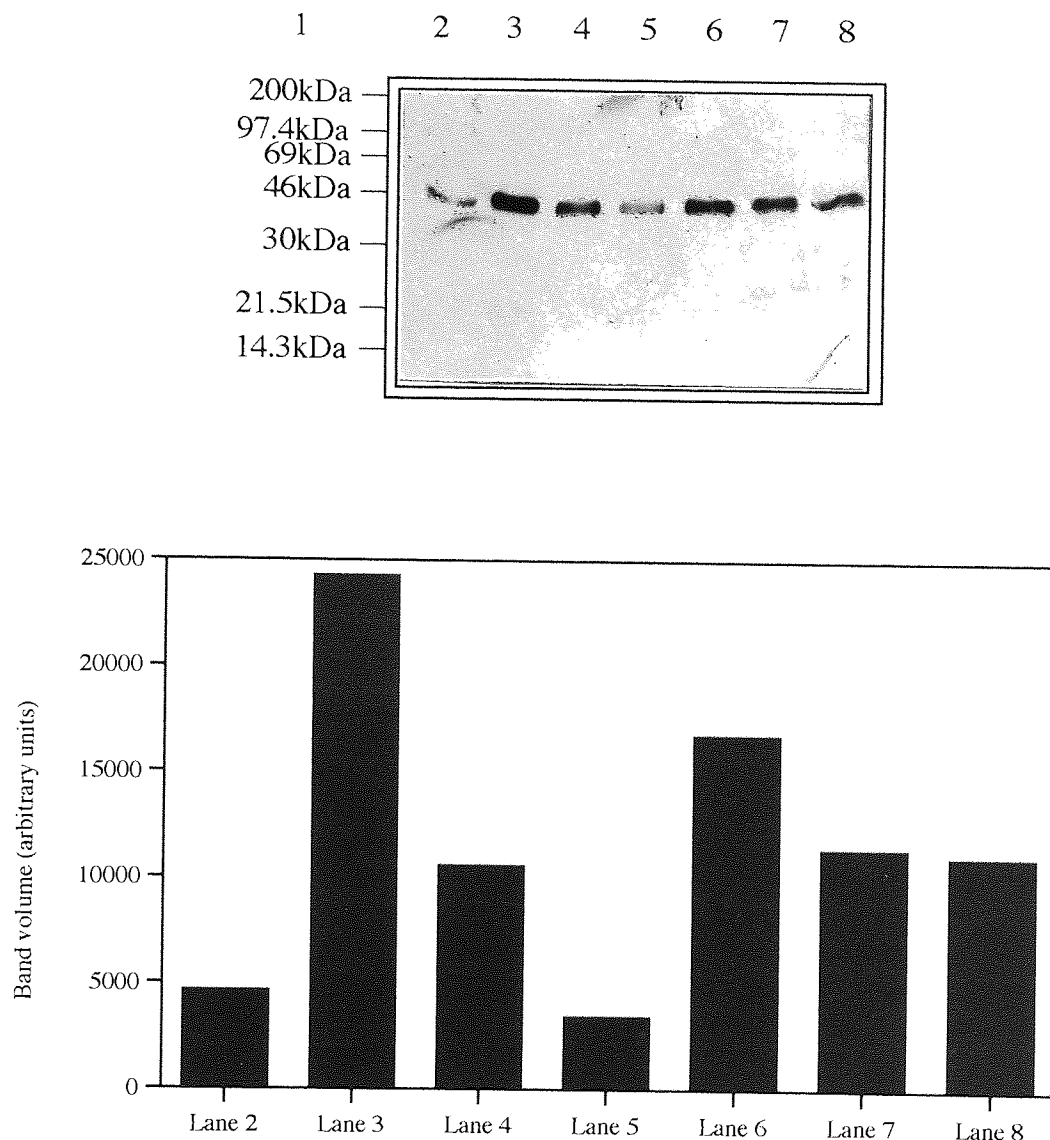


Figure 5.4.5.1 Adipocyte plasma membranes were isolated from normal NMRI mice (Lane 2) and mice bearing the MAC16 colon adenocarcinoma who exhibited 0% (Lane 3), 5% (Lane 4), 10% (Lane 5), 15% (Lane 6) and 20% (Lane 7) weight loss, and from mice with 10% weight loss who had received a single dose of EPA (1.25g/kg) (Lane 8). Lane 1 - molecular weight markers (A). 5 μ g of membrane protein was loaded for each sample. Band volume was measured as an indication of the quantity of Gα_i present (B).

Immunodetection of $G\alpha_s$ in White Adipocyte Plasma Membranes Isolated from Cachectic MAC16 Tumour-bearing Mice

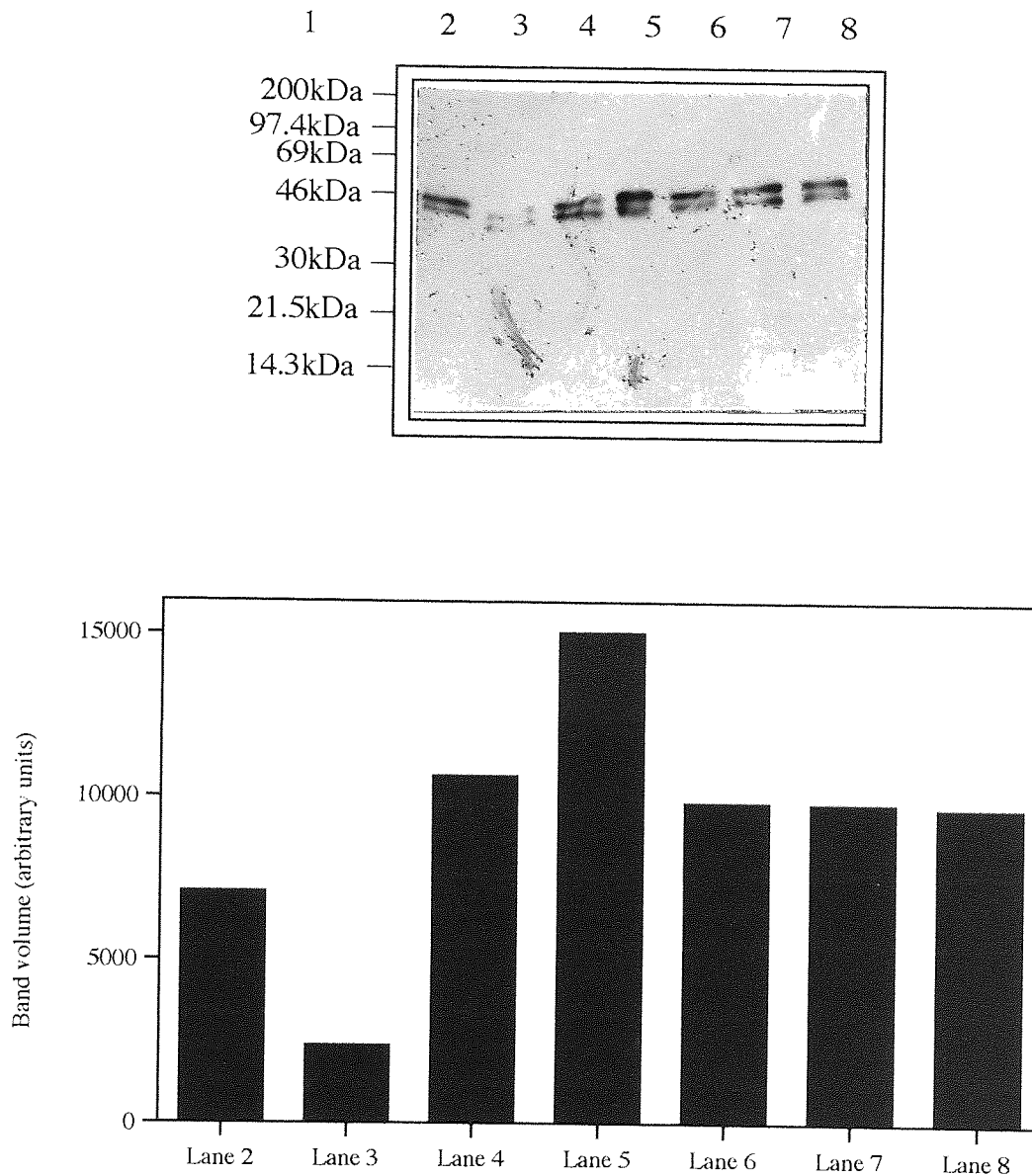


Figure 5.4.5.2 Adipocyte plasma membranes were isolated from normal NMRI mice (Lane 2) and mice bearing the MAC16 colon adenocarcinoma who exhibited 0% (Lane 3), 5% (Lane 4), 10% (Lane 5), 15% (Lane 6) and 20% (Lane 7) weight loss, and from mice with 10% weight loss who had received a single dose of EPA (1.25g/kg) (Lane 8). Lane 1 - molecular weight markers (A). 5 μ g of membrane protein was loaded for each sample. Band volume was measured as an indication of the quantity of $G\alpha_s$ present (B).

*Ratio of $G\alpha_s$ to $G\alpha_i$ Present in White Adipocyte Plasma Membranes
Isolated from Cachectic MAC16 Tumour-bearing Mice*

Sample	$G\alpha_s$ (arbitrary units)	$G\alpha_i$ (arbitrary units)	$G\alpha_s:G\alpha_i$
NMRI	7085.50	4633.00	1.53
MAC16 0%	2377.97	24286.50	0.10
MAC16 5%	10653.00	10568.00	1.00
MAC16 10%	15061.00	3452.00	4.36
MAC16 15%	9839.50	16752.00	0.59
MAC16 20%	9813.00	11378.00	0.86
EPA dosed	9685.28	11018.00	0.88

Table 5.4.5.1 The density of each band present on the Western blots illustrated in Figures 5.4.5.1 and 5.4.5.2 was determined as band volume using Gel Works Intermediate. The ratio of $G\alpha_s$ to $G\alpha_i$ in each sample was calculated as an estimate of the relative amount of each G-protein present in adipocyte plasma membranes at various stages in the progression of the cachectic state, and after the administration of EPA.

5.5 Discussion

The ability of EPA to inhibit the induction of lipolysis by the MAC16 tumour-derived LMF was first reported by Tisdale and Beck in 1991, and postulated to be the consequence of an attenuation of the increase in intracellular cAMP observed in adipocytes exposed to this factor. Subsequently, LMF has been demonstrated to induce body weight loss when administered intravenously to both NMRI and obese mice, due to a depletion of host adipose tissue stores only (Hirai *et al*, submitted for publication). In addition, there is a correlation between the extent of the cachectic state and the quantity of lipolytic activity detectable in either serum or urine (Groundwater *et al*, 1990). Furthermore, plasma levels of LMF were reported to decline in those cancer patients who responded positively to the administration of chemotherapy (Beck *et al*, 1990b). Hence there would appear to be a definite relationship between tumour production of LMF and the development and progression of cachexia.

Oral administration of the n-3 PUFA EPA has been reported to suppress the synthesis of IL-1 and TNF- α by mononuclear cells (Caughey *et al*, 1996; Endres, 1996; Meydani, 1996) and IL-6 by PBMCs (Wigmore *et al*, 1997c). Furthermore, EPA has been observed to attenuate the progression of IL-6-dependent cachexia (Ohira *et al*, 1996), a finding which further verifies the regulatory role of fatty acids upon cytokine production reported by Purasiri *et al* (1994). Therefore, in addition to exhibiting modulatory effects upon the intracellular accumulation of cAMP in adipocytes exposed to LMF, it is possible that EPA may act directly upon the tumour to down-regulate LMF production. Such down-regulation would reduce the concentration of circulatory LMF, hence diminishing the induction of lipolysis in host adipose tissue stores, and arresting the progression of host wasting. This hypothesis was investigated by purifying LMF from MAC16 tumours excised from mice which had received 1.25g/kg EPA for 24h and comparing its lipid-mobilising activity with that of LMF derived from undosed MAC16 tumour-bearing mice. No differences were observed between the induction of lipolysis by factor derived from either group of animals, therefore, production of LMF by the MAC16 tumour appeared to

be unaltered by EPA administration, indicating that the anti-cachectic effects of this fatty acid may be disparate from its anti-tumour effect, and that the primary anticachectic actions of EPA do not involve a modulation of factor production by the tumour itself. Attempts to further confirm this hypothesis, by immunoblotting of plasma samples taken from cachectic pancreatic cancer patients before and during dietary supplementation with EPA, in the form of MaxEPA capsules, were unsuccessful due to a non-specific interaction of the anti-p24 MAb with immunoglobulins present in plasma samples. Thus it would appear probable that the primary mechanism of action of EPA in attenuating the development of the cachectic state is an inhibition of the end organ effects of LMF, rather than a prevention of LMF production by the tumour.

Previously, Adamson (1992) reported a significant inhibition of isoprenaline-stimulated AC activity in rat adipocytes by EPA, DHA and AA. The ability of all three PUFAs to significantly attenuate the induction of lipolysis and AC activity by isoprenaline and LMF in murine white adipocytes has been confirmed. The inhibition of lipolysis by EPA was noted to be greater than that obtained using DHA, whilst AA appeared to be as efficacious, *in vitro*, as EPA. Similarly, EPA produced a more profound inhibition of AC activity than DHA, whilst AA, like EPA, could be observed to virtually abolish the stimulatory effects of LMF.

In addition, it could be observed that the inhibitory actions of EPA and AA, but not DHA, were dependent upon the degree of stimulation of lipolysis by LMF, with more profound attenuation occurring when adipocytes were exposed to higher concentrations of LMF. Hence, it should be noted that, the concentration of LMF employed in the stimulation of lipolysis or adenylate cyclase activity may affect the apparent actions of EPA. Given that the LMF utilised in all studies was not 100% pure, the presence of variable amounts of impurities necessitated the utilisation of seemingly varying concentrations of LMF, depending upon the dose response profile for each individual batch of purified material.

Therefore, it is possible that a greater degree of inhibition of lipolysis or AC activity by EPA may have been observed if a greater concentration of LMF had been utilised. It has been reported that LMF demonstrates sequence homology and identical gel migratory and antibody reactivity properties as Zn α_2 gp (Todorov *et al*, submitted for publication). The utilisation of pure recombinant Zn α_2 gp will enable verification of a putative role for this factor in cachexia, and also fuller quantification of the comparative effects of EPA, DHA and AA upon lipolysis and AC activity.

Adipocytes isolated from mice which have received oral EPA (1.25g/kg) have been demonstrated to display an attenuated degree of responsiveness to the catabolic actions of both isoprenaline and LMF (Khan, 1996). The effects of oral EPA upon AC were investigated utilising forskolin (7 β -Acetoxy-8,13-epoxy-1 α ,6 β ,9 α -trihydroxy-labd-14-en-11-one), a diterpene derived from the plant *Coleus forskohlii*, which has been demonstrated to increase cAMP levels via a direct activation of AC. A significantly reduced response to forskolin was manifest in membranes from EPA dosed mice, indicating a direct inhibition of AC by EPA, perhaps as the result of binding of EPA to AC and hence covalent modification of the enzyme. Indeed it has been reported that many of the enzymes involved in signal transduction may be regulated, either positively or negatively, by fatty acids (Sumida *et al*, 1993), with, for example, PKC activity in macrophages being decreased upon administration of EPA (Erickson *et al*, 1995). Hence, the induction of conformational or functional alterations of AC by the binding of PUFAs may be sufficient to attenuate the responsiveness of this enzyme to lipolytic stimuli.

The possible involvement of G-proteins in the attenuation of lipolysis and AC activity by fatty acids was first proposed by Adamson (1992) who reported an inhibition of pertussis toxin catalysed ADP-ribosylation of G α_i by EPA. The ability of fatty acids to covalently modify G-protein α sub-units has been noted (Hallak *et al*, 1994; Offermanns and Simon, 1996), and it is possible, therefore, that the displacement of palmitate from the N-terminus of G α_s and its replacement by EPA could affect the ability of G α_s to transfer signals

induced by ligand-receptor binding to AC, preventing the accumulation of cAMP, the sequential phosphorylation of PKA and HSL and hence the induction of lipolysis. Alternatively, binding of EPA to $G\alpha_i$ may stimulate this inhibitory G-protein, preventing activation of AC by $G\alpha_s$ and inhibiting the lipid-mobilising actions of isoprenaline and LMF.

Confirmation of the role of $G\alpha_i$ in the inhibition of lipolysis by EPA was provided by the observation that pre-treatment of isolated adipocytes, or adipocyte plasma membranes, with pertussis toxin prevented the inhibitory actions of EPA via an inactivation of $G\alpha_i$. This implies a direct interaction of EPA with $G\alpha_i$, which promotes the binding of this inhibitory G-protein sub-unit to AC, and hence hinders the conversion of ATP to cAMP, an occurrence which would obstruct the normal phosphorylation cascade associated with lipolysis, inhibiting the catabolic actions of the tumour-derived LMF. Interestingly, pre-treatment of adipocytes with pertussis toxin failed to abolish the inhibition of isoprenaline or LMF-stimulated lipolysis associated with DHA. Therefore, it would appear that the mechanism(s) responsible for the inhibition of lipolysis and AC activity by DHA are different from those associated with EPA, implying that alterations in membrane fluidity alone are insufficient to account for the inhibitory actions of EPA. The role of AA in the inhibition of lipolysis and AC activation remains unclear. Whilst it would appear that an interaction of AA with AC is sufficient to diminish isoprenaline-stimulated lipolysis, $G\alpha_i$ may be implicated in the inhibition of LMF-induced lipid-mobilisation. Such contradictory findings are difficult to explain, and a fuller investigation of the role of AA in the attenuation of lipolysis and AC activity is necessary in order to clarify the underlying mechanism(s).

A concurrent up-regulation of the expression of $G\alpha_{i1}$, $G\alpha_{i2}$ and $G\alpha_{i3}$, but not $G\alpha_s$, has been noted in adipocytes isolated from hypothyroid rats (Milligan and Saggerson, 1990) indicating an increased efficacy of anti-lipolytic agents such as adenosine, and correlating to the gain in body weight often observed in individuals exhibiting hypothyroidism.

Likewise, the androgen testosterone has been observed to influence G-protein expression in fat cells, with its depletion by castration inducing a down-regulation of both $G\alpha_s$ and $G\alpha_{i1-2}$, and testosterone replacement restoring $G\alpha_s$ expression (Dieudonne *et al*, 1993). In addition, mutations have been observed to occur within the α_s genes of some tumours, resulting in persistent stimulation of AC activity via a block of α_s in the active GTP-bound conformation (Vallar, 1996). Such activation may induce cellular proliferation, conversely, mutational activation of α_{i2} has been implicated in tumour suppression via a phosphorylation of the tumour suppressor gene *Rb*. Hence, in addition to agonist-mediated receptor expression, notably the up-regulated expression of the human β_3 -adrenoceptors due to an interaction between cAMP and the promoter of the relevant gene (Langin *et al*, 1995), or administration of exogenous leptin to leptin-deficient, hypometabolic *ob/ob* mice demonstrating reduced adipocyte β_3 -adrenoceptor levels (Breslow *et al*, 1997), adipocyte response to lipolytic stimuli also depends upon the expression and conformation of G-proteins. Alterations in adipocyte G-proteins were observed with the progression of the cachectic state. These consisted principally of a reduction of membrane $G\alpha_i$ expression allied with increased $G\alpha_s$, changes which would favour mobilisation of lipid stores from adipocytes, and hence facilitate host tissue catabolism. Such alterations were noted in mice manifesting up to 10% loss of their original body weight, and correlated with maximal tumour production of LMF. The subsequent decrease in $G\alpha_s$ expression and increase in $G\alpha_i$ may reflect diminished tumour production of LMF. Hence it seems probable that it is the presence of circulatory LMF which is responsible for the observed alterations in G-protein expression, and that a reduction in LMF levels permits a normalisation of membrane G-proteins in a similar fashion to the testosterone-influenced alterations noted by Dieudonne *et al* (1993). Short term (24h) administration of EPA (1.25g/kg) markedly altered adipocyte $G\alpha_i$ and $G\alpha_s$ contents, enhancing $G\alpha_i$ and diminishing $G\alpha_s$. Such an observation could, at least in part, account for the stabilisation of host adipose tissue stores recorded in weight-losing pancreatic cancer patients receiving dietary supplementation with EPA (Wigmore *et al*, 1996a).

In addition, the observed up-regulated responsiveness to isoprenaline and LMF demonstrated by adipocytes isolated from cachectic MAC16 tumour-bearing mice, and the concomitant decrease in sensitivity upon administration of EPA, may also be the consequence of alterations in adipocyte G-protein expression or activity. The possibility of an elevation of adipocyte AC inducing such enhanced responsiveness was discounted by Khan (1996) who demonstrated an unaltered degree of forskolin-stimulated cAMP formation in adipocytes derived from cachectic mice, and thus postulated that an alteration in either receptor, or G-protein, expression or activity must be responsible for this phenomenon.

The observation of variations in the amount of $G\alpha_i$ and $G\alpha_s$ present within isolated white adipocyte plasma membranes would appear to imply that any up-regulation of adipocyte sensitivity to a tumour-derived catabolic factor may be the consequence of alterations in G-protein expression which favour the induction of host wasting. As yet, the occurrence of such alterations in human cancer patients has not been investigated. However, it is possible that white adipocytes isolated from cachectic cancer patients may also demonstrate enhanced responsiveness to LMF, and that this may be the consequence of an up-regulation of membrane $G\alpha_s$ allied with a concomitant down-regulation of $G\alpha_i$. The changes in G-protein expression noted upon EPA administration imply an inhibition of tumour-induced host catabolism via a repression of the efficacy of LMF. Hence adipocyte plasma membranes prepared from cachectic mice who had received EPA could be expected to demonstrate the same degree of sensitivity to lipolytic stimuli as membranes from non tumour-bearing animals. Similar mechanism(s) may underlie the attenuation of the cachectic state observed in EPA supplemented cancer patients. This hypothesis, however, requires confirmation.

5.6 Conclusions

The results reported in Section 5.2 verify the findings of Raclot *et al* (1994); Leray *et al* (1995); Leaf *et al* (1995) and Layne *et al* (1996), namely that oral administration of PUFAs induces significant alterations in the fatty acid profile of plasma lipoproteins and white and brown adipose tissue. Oral administration of EPA to weight-losing advanced pancreatic cancer patients, in the form of either MaxEPA capsules, pure EPA capsules or a fish oil-containing dietary supplement, resulted in significantly increased plasma levels of EPA. Incorporation of EPA into adipose tissue of these patient groups was not determined, however, EPA could be observed to be incorporated into the plasma, adipose tissue and skeletal muscle of orally dosed mice, hence it may be assumed that similar alterations probably occur in EPA supplemented cancer patients. In addition, an incorporation of exogenous fatty acids into adipocyte plasma membranes *in vitro* was observed.

The therapeutic potential of EPA in cachexia was confirmed by Wigmore *et al* (1996a; 1996b) who recorded a stabilisation of body weight, adipose tissue stores and skeletal muscle in recipient patients. The mechanisms of action associated with these observations, however, remained unclear. Several workers have documented the ability of n-3 PUFAs to down-regulate the production of various cytokines (Purasiri *et al*, 1994; Caughey *et al*, 1996; Endres *et al*, 1996; Meydani *et al*, 1996; Wigmore *et al*, 1997c). The results reported in Section 5.4.2, however, reveal that whilst short term administration of EPA is sufficient to modulate the induction of AC activation by a MAC16 tumour-derived LMF, it is incapable of attenuating the production of this LMF by the MAC16 tumour. Hence, it seems probable that EPA exerts its anti-cachectic actions via an inhibition of the end organ effects of tumour-derived catabolic factor(s), rather than an attenuation of the production of such factors by cachexigenic tumours

Some binding of EPA to AC would appear to account, at least in part, for the inhibitory actions of this n-3 PUFA (Section 5.4.2), whilst studies utilising pertussis toxin (Section 5.4.3) imply an interaction between EPA and inhibitory G-protein subunits. Stimulation of

$G\alpha_i$ would inhibit the intracellular accumulation of cAMP and hence obviate the phosphorylation and activation of HSL, the rate limiting enzyme of the lipolytic cascade. Preliminary studies into the expression of $G\alpha_i$ and $G\alpha_s$ by white adipocyte plasma membranes during the progression of the cachectic state revealed alterations which would appear to favour the catabolic actions of tumour-derived LMF, namely a down-regulation of $G\alpha_i$ and an up-regulation of $G\alpha_s$, when tumour factor production was maximal, that is approximately 16% loss of original body weight, which was seemingly reversed upon EPA administration. These alterations may occur as the result of long term exposure to tumour-derived factors, inducing a state of augmented sensitivity to catabolic stimuli, and such alterations are evidently reversed by administration of EPA. Hence, in addition to preventing AC activation and stimulating $G\alpha_i$ it is possible that EPA acts at the level of the gene to affect G-protein expression. Analysis of $G\alpha_i$ and $G\alpha_s$ mRNA expression in both murine and human adipose tissue is necessary to confirm the hypothesis that EPA promotes membrane expression of $G\alpha_i$ whilst suppressing $G\alpha_s$, and to determine whether the alterations in G-protein expression observed with disease progression are unique to cachexigenic tumours or also occur in the presence of non-cachexigenic tumours. However, the observation of an increased sensitivity to LMF by adipocytes isolated from cachectic mice (Khan, 1996), allied with the observed normalisation of this response upon EPA administration (Section 5.4.2) may lend support to this hypothesis.

Chapter 6 - Discussion and Conclusions

Eicosapentaenoic Acid, a Modulator of Signal Transduction.

Cancer cachexia has been postulated to be the result of the catabolic actions of two tumour-derived factors, PIF and LMF, which mobilise proteins from skeletal muscle and lipids from adipose tissue, respectively. The relative significance of LMF as a cachectic mediator is unclear, given that when administered *in vivo*, PIF is capable of inducing alterations in body composition due to loss of both muscle mass and adipose tissue stores (Cariuk *et al*, 1997). However, LMF has also been demonstrated to reduce host adipose tissue stores when administered intravenously (Beck *et al*, 1990a; Todorov *et al*, unpublished results), and to mobilise TAG from isolated murine white adipocytes via an interaction with a β -adrenoceptor and the subsequent phosphorylation and activation of PKA (Khan, 1996), in a manner similar to that of naturally occurring lipolytic hormones.

Over stimulation of β -adrenoceptors by the presence of excessive concentrations of agonist is typically prevented by desensitisation. Rapid desensitisation is often the consequence of receptor phosphorylation and uncoupling of $G\alpha_s$ by PKA and β -adrenergic receptor kinase (β ARK). Sustained receptor activation induces degradation of receptor protein and destabilisation of receptor mRNA, hence reducing total receptor numbers (Strosberg, 1996). Interestingly, however, the β_3 -adrenoceptor appears completely resistant to short term desensitisation, probably due to the absence of the PKA and β ARK phosphorylation consensus sites from the third intracellular domain and the C-terminal region of the receptor (Strosberg, 1996). The evident lack of receptor desensitisation, or down-regulation, in adipocytes isolated from mice which have undergone long term exposure to LMF lead Khan (1996) to postulate a role for the β_3 -adrenoceptor in the mobilisation of host lipid stores associated with cachexia.

The coupling of β -adrenoceptors to AC is dependent upon G-proteins, with $G\alpha_s$ potentiating, and $G\alpha_i$ inhibiting, cAMP formation. The involvement of $G\alpha_i$ in the inhibitory actions of EPA upon the induction of lipolysis by the MAC16 tumour-derived LMF was postulated by Adamson (1992) and confirmed by this study. Such evidence further implies a role for the β_3 -, or an additional novel β -adrenoceptor, since whilst β_1 -, β_2 - and β_3 -adrenoceptors have been demonstrated to interact with $G\alpha_s$, thus far only β_3 -adrenoceptors have been suggested to also couple with $G\alpha_i$ (Strosberg and Pietri-Rouxel, 1996).

Studies employing a β -adrenoceptor antagonist, SR 59230A, which exhibits an approximately ten fold greater affinity for β_3 - over β_2 -adrenoceptors, provided further corroboration of the participation of β_3 -adrenoceptors in the induction of lipolysis by LMF (Chapter 4.1). These findings may appear to imply a lack of efficacy of LMF in human adipocytes due to the relative paucity of functional β_3 -adrenoceptors (Lafontan and Berlan, 1993; Giacobino, 1995), hence intimating that LMF may play no role in the development and progression of clinical cancer cachexia. The results reported in Chapter 4.2, however, verify the ability of both murine and human LMF to induce lipolysis in isolated human white omental adipocytes. Furthermore, a certain degree of species specific interaction of LMF with its receptor is manifested, suggesting that whilst murine and human LMF may be comparable, they are not completely homologous. It appears probable, however, that both human and murine LMF are responsible for the atrophy of host adipose tissue deposits observed in cachexia due to the induction of lipolysis via an interaction with a β_3 -adrenoceptor. This interaction initiates the binding of GTP to $G\alpha_s$ and hence activation of AC and the associated signal transduction cascade.

The apparent increase in responsiveness of adipocytes isolated from cachectic mice, to LMF *in vitro*, observed by Khan (1996) implies an up-regulation of either receptor numbers or stimulatory G-protein expression. Immunoblotting of adipocyte plasma membranes revealed changes in G-protein expression with the progression of the cachectic

state, suggesting some tumour-induced alteration. Modifications of G-protein expression have been observed to occur in various disease states, including hypothyroidism (Milligan and Saggerson, 1990), and so it is possible that a similar scenario may occur during host wasting.

Cachexigenic tumours have been demonstrated to provoke profound metabolic adaptations to occur in the host, inducing a catabolic state whereby mobilised proteins and lipids are utilised by the tumour to support its metabolic and structural requirements. As previously stated, such atrophy of host tissues is made possible by the secretion from the tumour of factors possessing the capacity to induce lipolysis and proteolysis, and it is possible that in addition to inducing host wasting, LMF may be capable of producing alterations in adipocyte G-protein expression, namely increased $G\alpha_s$ and decreased $G\alpha_i$, which facilitate the catabolic actions of this factor. Such elevated expression of $G\alpha_s$ during the progression of the cachectic state would account for the up-regulated response to LMF demonstrated by adipocytes and adipocyte plasma membranes isolated from cachectic mice.

Thus, it may be concluded that the signal transduction pathway associated with lipolysis in cachexia is potentiated by its stimulatory factor, namely LMF. Hence, in addition to inducing TAG mobilisation from adipocytes, it would appear that LMF is also capable of inducing alterations in adipocyte $G\alpha_s$ and $G\alpha_i$ expression, probably via an alteration of G-protein mRNA, such that the actions of LMF upon the adipocyte are enhanced.

Polyunsaturated fatty acids have been demonstrated to possess the ability to modify a variety of biological processes. One such area of influence involves cellular membranes and the associated proteins involved in the transduction of signals induced by the binding of a ligand to its receptor. Alterations in plasma membrane fluidity, due to membrane incorporation of either n-3 or n-6 PUFAs have been suggested to be sufficient to modulate the expression of membrane bound molecules such as intercellular adhesion molecule-1 (Lu *et al*, 1995). However, such effects are non-specific, occurring upon incorporation of

a number of PUFAs, not just EPA, whilst the progression of the cachectic state in mice bearing the experimental MAC16 tumour was attenuated by EPA alone, with oral administration of LA and DHA being ineffective (Tisdale and Beck, 1991). In addition, the apparently disparate mechanisms associated with the inhibition of LMF-induced lipolysis by EPA and DHA suggests that simple alterations in membrane fluidity are insufficient to account for the observed effects.

The attenuation of cancer cachexia by EPA has been demonstrated in both mice bearing an experimental cachexigenic tumour model (Tisdale and Dhesi, 1990) and human cancer patients (Wigmore *et al*, 1996a; Wigmore *et al*, 1996b), and has been noted to be accompanied by a stabilisation of body weight, skeletal muscle mass and subcutaneous adipose tissue (Wigmore *et al*, 1996a). The mechanisms underlying this phenomenon have been investigated in Chapter 5 of this report. Adamson (1992) noted a decreased ADP-ribosylation of adipocyte plasma membrane $G\alpha_i$ by pertussis toxin in membranes pre-treated with EPA, hence some interaction of EPA and the signal transduction pathway associated with LMF was implied. The covalent modification of G-protein α sub-units by fatty acids has been documented (Muszbek and Laposata, 1993; Hallak *et al*, 1994), as have the interactions which occur between fatty acids and almost all of the enzymes associated with signal transduction (Sumida *et al*, 1993). Both membranes prepared from mice orally dosed with EPA, and membranes exposed to exogenous EPA *in vitro* demonstrated an attenuated activation of AC in response to LMF, whilst membranes isolated from EPA dosed mice also revealed a reduced responsiveness to forskolin, indicating some direct interaction between AC and EPA. Covalent modification, and stimulation, of $G\alpha_i$ by EPA, but not DHA, was also demonstrated to contribute to the modulatory actions of this n-3 PUFA. The attenuated responsiveness of adipocyte plasma membranes prepared from cachectic mice which have received a single dose of 1.25g/kg EPA 24h prior to sacrifice, to LMF suggests that EPA may act via a reversal of the alterations induced in adipocytes by the cachectic state. Immunoblotting of such plasma

membranes revealed a down-regulation of $G\alpha_s$ expression allied with increased $G\alpha_i$ expression, hence EPA reverses the potentiation of lipolysis associated with LMF.

Such alterations have, thus far, only been studied short term, that is over 24h, and whilst the end organ effects of LMF may be seen to be attenuated, tumour production of this catabolic factor appears unaltered. Previously Beck *et al* (1990b) noted an alteration in serum lipolytic activity of cancer patients with response to chemotherapy, presumably as the consequence of the anti-tumour effects of the drug. It has been proposed that EPA is primarily anti-cachectic, however, long term inhibition of the catabolic effects of LMF, or PIF, by EPA may ultimately deprive the tumour of the nutrients it requires for metabolism and hence reduce tumour factor production, implying that long term EPA may also induce changes in serum lipolytic activity, a hypothesis in need of investigation. The effects of longer term administration of EPA to cachectic mice require clarification in order to determine whether or not additional changes in G-protein levels occur, and the ultimate effects upon LMF production by the tumour. A full investigation is also required in order to ascertain the mechanism(s) underlying the preservation of skeletal muscle mass which occurs upon EPA administration, and to determine whether this also primarily depends upon an inhibition of the signal transduction pathway associated with PIF. However, thus far, it appears that the anti-lipolytic mechanism of action of EPA relies primarily on an end organ inhibition of the catabolic effects of tumour-derived factors.

Such modulation by EPA of the signal transduction pathway associated with the induction of lipolysis by tumour-derived LMF appears to be specific to that fatty acid, and not merely the consequence of an increased membrane fluidity due to the incorporation of long chain PUFAs, since whilst DHA interacts with AC alone, EPA interacts with AC and $G\alpha_i$. In addition, EPA has been demonstrated to affect G-protein expression and so may specifically interact with those areas of DNA responsible for the promotion or suppression of $G\alpha_i$ and $G\alpha_s$ mRNA transcription, hence modulating adipocyte plasma membrane $G\alpha_i$ and $G\alpha_s$ expression. The observed inhibition of cell growth (Falconer *et al*, 1994c) and

induction of apoptosis (Lai *et al*, 1996) in pancreatic cancer cell lines exposed to EPA implies some interaction between this fatty acid and the genetic mechanisms responsible for the integrity and proliferation of neoplastic cells. In addition, nuclear translocation of the transcription factor nuclear factor κ B (NF- κ B) and its binding to DNA have been suggested to be influenced by PUFAs (Camandola *et al*, 1996). Furthermore, dietary PUFAs have been reported to regulate the synthesis of hepatic enzymes via either an interference with the translation rate of mRNA, or a reduction in the amount of mRNA encoding for the enzyme (Clarke and Jump, 1994). Indeed, it has even been postulated that the two principal sites by which PUFAs, or their metabolites, control hepatocyte functioning are either a rapid effect upon gene expression, or an enrichment of membrane lipids inducing alterations in signal transduction (Clarke and Jump, 1994). Thus the ability of dietary PUFAs to modulate gene expression has been demonstrated.

In order to verify that such alterations in G-protein mRNA are occurring, Northern blotting of plasma membrane samples from tumour-bearing mice with varying degrees of weight loss, and from EPA dosed mice is necessary. In addition, it would be useful to investigate the consequences of *in vivo* administration of DHA and other EFAs upon membrane $G\alpha_i$ and $G\alpha_s$ levels, in order to confirm the specificity of the EPA-induced alterations observed in this study.

Concluding Remarks

In conclusion, this study confirms a putative role for LMF in both the development and progression of cachexia in human cancer patients. Both LMF derived from human urine, and murine tumours, induced lipolysis in isolated human white omental adipocytes in a dose-dependent fashion, via an activation of AC (Hirai *et al*, submitted for publication).

The n-3 PUFA EPA has been demonstrated to induce significant alterations in plasma phospholipid, adipose tissue and skeletal muscle fatty acid composition when administered

orally. Furthermore, EPA attenuated LMF-induced lipolysis via both an apparent down-regulation of $G\alpha_s$ expression, and an up-regulation of $G\alpha_i$ expression, in contrast to the alterations in adipocyte G-protein levels observed during the progression of the cachectic state. In addition EPA exhibited a direct interaction with AC, and an activation of $G\alpha_i$, both of which inhibited the catabolic actions of LMF upon the adipocyte.

Therefore, the mechanism(s) underlying the attenuation of cancer cachexia by oral administration of the n-3 PUFA EPA involve, at least in part, a direct inhibition of the end organ effects of a tumour-derived catabolic factor, LMF. This is most likely the consequence of the ability of EPA to modulate both the signal transduction pathway associated with this factor, and to influence G-protein expression, possibly via alterations in G-protein mRNA, an hypothesis requiring clarification.

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Appendix 1

Amino acids

Code letter	Abbreviation	Amino acid
A	Ala	Alanine
C	Cys	Cysteine
D	Asp	Aspartic acid
E	Glu	Glutamic acid
F	Phe	Phenylalanine
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
K	Lys	Lysine
L	Leu	Leucine
M	Met	Methionine
N	Asn	Asparagine
P	Pro	Proline
Q	Gln	Glutamine
R	Arg	Arginine
S	Ser	Serine
T	Thr	Threonine
V	Val	Valine
W	Trp	Tryptophan
Y	Tyr	Tyrosine

Appendix 2

Publications

Papers

Investigations into the mechanism of catabolism of adipose tissue by a tumour-produced lipid mobilising factor. S Khan, SA Price, MJ Tisdale (1997). Submitted for publication.

Biological evaluation of a lipid-mobilising factor (LMF) isolated from the urine of cancer patients. K Hirai, HJ Hussey, MD Barber, SA Price, O Ishiko, MJ Tisdale. Submitted for publication.

Mechanism of inhibition of a tumour lipid-mobilising factor by eicosapentaenoic acid. SA Price, MJ Tisdale. In preparation.

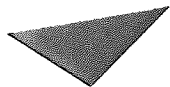
Abstracts

The role of eicosapentaenoic acid in the attenuation of cancer cachexia. S Khan, SA Price, MP Wynter, MJ Tisdale. BACR 37th Annual Scientific Meeting, Edinburgh, 1996.

Elucidation of the signal transduction pathway of a tumour-derived lipid-mobilising factor. S Khan, SA Price, MJ Tisdale. BACR 38th Annual Scientific Meeting, Southampton, 1997.

Inhibition of the signal transduction pathway of a tumour-derived cachectic factor by eicosapentaenoic acid. SA Price and MJ Tisdale. 4th International Congress on Essential Fatty Acids and Eicosanoids, Edinburgh, 1997.

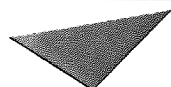
THE ROLE OF EICOSAPENTAENOIC ACID IN THE ATTENUATION OF CANCER
CACHEXIA, S. Khan*, S.A. Price*, M.P. Wynter, M.J. Tisdale, Pharmaceutical
Sciences Institute, Aston University, Birmingham B4 7ET.



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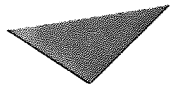
ELUCIDATION OF THE SIGNAL TRANSDUCTION PATHWAY OF A TUMOUR-DERIVED LIPID-MOBILISING FACTOR S Khan, SA Price*, MJ Tisdale, Pharmaceutical Sciences Institute, Aston University, B'ham B4 7ET.



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Inhibition of the signal transduction pathway of a tumour-derived cachectic factor by eicosapentaenoic acid SA Price, MJ Tisdale, Pharmaceutical Sciences Institute, Aston University, Birmingham B4 7ET, United Kingdom.



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Appendix 3

Determination of Specific Activity of LMF

In an attempt to standardise the concentration of LMF utilised from different purification batches the specific activity of the various preparations of LMF was determined via dose response assays for both lipolysis and adenylate cyclase activity. Protein concentrations relating to sub-optimal stimulation of adipocytes were noted, and utilised in further studies. Typical dose responses obtained for the induction of (A) lipolysis and (B) adenylate cyclase activity by a single batch of LMF are illustrated below. Protein concentrations of $20\mu\text{g/ml}$ and $15\mu\text{g}/100\mu\text{l}$, respectively, were employed in subsequent experiments.

