Mechanism of Attenuation of Skeletal Muscle Protein Catabolism in Cancer Cachexia by Eicosapentaenoic Acid[1]

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ABSTRACT

Cancer cachexia is characterized by selective depletion of skeletal muscle protein reserves. Soleus muscles from mice bearing a cachexia-inducing tumor (MAC16) showed an increased protein degradation *in vitro*, as measured by tyrosine release, when compared with muscles from nontumor-bearing animals. After incubation under conditions that modify different proteolytic systems, lysosomal, calcium-dependent, and ATP-dependent proteolysis were found to contribute to the elevated protein catabolism. Treatment of mice bearing the MAC16 tumor with the polyunsaturated fatty acid, eicosapentaenoic acid (EPA), attenuated loss of body weight and significantly suppressed protein catabolism in soleus muscles through an inhibition of an ATP-dependent proteolytic pathway. The ATP-ubiquitin-dependent proteolytic pathway is considered to play a major role in muscle catabolism in cachexia, and functional proteasome activity, as determined by "chymotrypsin-like" enzyme activity, was significantly elevated in gastrocnemius muscle of mice bearing the MAC16 tumor as weight loss progressed. When animals bearing the MAC16 tumor were treated with EPA, functional proteasome activity was completely suppressed, together with attenuation of the expression of 20S proteasome α-subunits and the p42 regulator, whereas there was no effect on the expression of the ubiquitin-conjugating enzyme ($E2_{14k}$). These results suggest that EPA induces an attenuation of the up-regulation of proteasome expression in cachectic mice, and this was correlated with an increase in myosin expression, confirming retention of contractile proteins. EPA also inhibited growth of the MAC16 tumor in a dose-dependent manner, and this correlated with suppression of the expression of the 20S proteasome α-subunits in tumor cells, suggesting that this may be the mechanism of tumor growth inhibition. Thus EPA antagonizes loss of skeletal muscle proteins in cancer cachexia by down-regulation of proteasome expression, and this may also be the mechanism for inhibition of tumor growth.

INTRODUCTION

Cancer patients with cachexia show a specific depletion of their skeletal muscle mass, whereas the visceral protein compartment remains unchanged (1). This loss of muscle protein forms part of the syndrome of cancer cachexia, which results in the loss of function of the cancer patient and eventually death from hypostatic pneumonia, attributable to the loss of respiratory function.

Most treatments that have been investigated to alleviate this terminal decline have not succeeded in attenuating the loss of muscle mass. Thus, administration of total parenteral nutrition resulted in a short-term weight gain, suggesting retention of water (2), whereas body composition analysis showed no preservation of lean body mass and a temporary maintenance of fat stores. The appetite stimulant megestrol acetate has also been shown to induce a weight gain of greater than 5% in 15% of the patients treated (3), although body composition analysis showed no evidence for an increase in lean body mass, and the weight gain appeared to arise from an increase in adipose tissue or an increase in body fluid (4). Another appetite stimulant, the serotonin antagonist cyproheptadine, failed to significantly abate progressive weight loss in cachectic patients (5). Thus, provision of excess calories alone is unable to counteract loss of skeletal muscle in cachexia.

These results suggest that metabolic alterations in cachexia are more important than anorexia in mediating loss of skeletal muscle. Whereas various cytokines have been postulated to be responsible for the metabolic changes in cachexia seen in some animal models, there is little evidence that they play a major role in human cancer cachexia (6). We have isolated a PIF[3] produced by cachexia-inducing murine (7) and human tumors (8), which is distinct from the cytokines and is able to induce protein catabolism directly in isolated skeletal muscle (9). PIF was found to be excreted in the urine of patients with carcinomas of the pancreas, liver, rectum, colon, breast, lung, and ovary where the weight loss was greater than or equal to 1 kg/month (10). PIF was detected in the urine of 80% of patients with pancreatic cancer ($n = 55$; Ref. 11). These patients had a significantly greater total weight loss and rate of weight loss than in patients without PIF in the urine. The activity of PIF is attenuated by the polyunsaturated fatty acid, EPA, in model systems (12), and EPA is able to prevent the weight loss induced by the MAC16 colon adenocarcinoma, with preservation of lean body mass (13). This effect is attributable to inhibition of the increased protein degradation seen in skeletal muscle.

EPA alone has been shown to attenuate the development of weight loss in pancreatic cancer patients (14) and, when incorporated into a nutritional supplement, produced significant weight gain (2.5 kg at 7 weeks; Ref. 15). Unlike nutritional supplementation alone, body composition analysis showed a significant gain in lean body mass with no change in fat mass or percentage body water. This suggests that an EPA-enriched nutritional supplement may be able to reverse cachexia in advanced pancreatic cancer. A recent clinical study (16) in patients with generalized solid tumors also showed EPA to prolong survival.

There are three major proteolytic pathways responsible for the catabolism of proteins in skeletal muscle: the lysosomal system, which is predominantly concerned with proteolysis of extracellular proteins and cell surface receptors (17); a cytosolic Ca^{2+}-activated system, which may play an important role in tissue injury, necrosis, and autolysis (18) and which is independent of ATP; and an ATP-ubiquitin-dependent proteolytic pathway, which is believed to be responsible for the breakdown of the bulk of the intracellular proteins in skeletal muscle (17). In the present study, the effect of EPA on each of the proteolytic pathways has been examined in skeletal muscle of mice bearing the MAC16 tumor, which produces profound cachexia with small tumor masses (19). Previous studies (20) have shown that the ATP-ubiquitin-dependent proteolytic pathway is responsible for the loss of skeletal muscle in such cachectic mice, and attention has been focused on this catabolic pathway.

MATERIALS AND METHODS

Animals. Pure strain female NMRI mice were obtained from our own inbred colony and were fed a rat and mouse breeding diet (Special Diet Services, Witham, United Kingdom) and water *ad libitum*. Animals were

[3] The abbreviations used are: PIF, proteolysis-inducing factor; EPA, eicosapentaenoic acid.

implanted s.c. in the flank with fragments of the MAC16 tumor by means of a trochar, selecting from donor animals with established weight loss (19). Weight loss was evident 10–12 days after transplantation, when the animals were randomized to receive EPA (0.5 and 2.5 g/kg/day) p.o. by gavage or olive oil as control. Nontumor-bearing animals of the same age and weight formed a control group. All of the groups contained 6–8 mice. Animals were treated at 0, 24, and 48 h, and body weight and tumor volume were monitored daily. They were terminated by cervical dislocation 2 h after the last dose. The soleus muscles were quickly dissected out, together with intact tendons, and maintained in isotonic ice-cold saline before determination of protein degradation.

Materials. Mouse monoclonal antibody to 20S proteasome subunits α 1, 2, 3, 5, 6, and 7 (clone MCP231) was purchased from Affiniti Research Products, Exeter, United Kingdom. Mouse monoclonal antibody to p42 was a gift from Dr. Jane Arnold. Rabbit polyclonal antisera to ubiquitin-conjugating enzyme E2 (anti-UBC2 antibody) was a gift from Dr. Simon Wing, McGill University, Montreal, Quebec, Canada. The antibody recognizes both isoforms of $E2_{14k}$ encoded by HHR6A and HHR6B (21). The *HHR6B* gene encodes the isoform for which mRNA levels increase in atrophying muscles. The antibody detected $E2_{14k}$ as a M_r 17,000 band. Mouse monoclonal antibody to myosin was purchased from Novacastra (Newcastle-upon-Tyne, United Kingdom). Peroxidase-conjugated rabbit antimouse antibody was purchased from Dako Ltd. (Cambridge, United Kingdom), and peroxidase-conjugated goat antirabbit antibody was purchased from Sigma Chemical Co., Dorset, United Kingdom.

Determination of the Mechanism of Muscle Protein Degradation. The freshly dissected soleus muscles were fixed via the tendons to aluminum wire supports, under tension, at approximately resting length to prevent muscle shortening and preincubated for 45 min in 3 ml of oxygenated (95% oxygen: 5%carbon dioxide) Krebs-Henseleit bicarbonate buffer (pH 7.4) containing 5 mM glucose and 0.5 mM cycloheximide. The protein degradation rate was determined during a further 2-h incubation by the release of tyrosine (22). For the evaluation of lysosomal proteolysis, the incubation medium contained 10 mM ammonium chloride, 250 μM chloroquine, 10 mM methylamine, and 30 μM leupeptin (23). Calcium-dependent proteolysis was determined by comparing the rate of tyrosine release from muscles incubated in medium containing 2.5 mM calcium with those incubated in calcium-depleted medium containing the calpain I and II inhibitor E-64 (100 μM; Ref. 24). The muscles were also incubated in the presence of 10 mM methylamine to inhibit lysosomal protein degradation. To study the role of energy-dependent proteolysis, muscles were depleted of intracellular ATP by a 1-h preincubation in medium containing 5 mM deoxyglucose and 0.2 mM sodium azide in the absence of glucose. The muscles were then incubated for an additional 2 h in the absence of calcium and with inhibition of lysosomal protein degradation, and the tyrosine released was compared with that from muscles incubated in the presence of 5 mM glucose. Thus, changes in tyrosine release reflect nonlysosomal, calcium-independent, energy-dependent proteolysis.

Measurement of Proteasome Activity. The chymotrypsin-like activity of the proteasome was determined fluorometrically according to the method of Orino *et al.* (25), with some modifications. Cells were washed twice with ice-cold PBS and scraped from the substratum into 20 mM Tris HCl (pH 7.5), 2 mM ATP, 5 mM MgCl$_2$, and 1 mM DTT (0.5 ml). The cells were dissociated by sonication with three pulses of 15 s with 10-s intervals at 4°C. The sonicate was then centrifuged for 10 min at 15,000 rpm at 4°C, and the resulting supernatant (0.1 ml) was used to determine chymotrypsin-like activity using the fluorogenic substrate succinyl-LLVY-MCA (0.1 mM) in a total volume of 0.2 ml of 100 mM Tris HCl (pH 8.0) for 1 h. The reaction was terminated by the addition of 80 mM sodium acetate (pH 4.3; 1 ml), and the fluorescence was determined with an excitation wavelength of 360 nm and an emission wavelength of 460 nm after further dilution with 2 ml 80 mM sodium acetate. The activity was adjusted for the protein concentration of the sample, determined using the Bradford assay (Sigma Chemical Co.).

Western Blot Analysis. Samples of gastrocnemius cytosolic protein (2.5 to 5 μg) were resolved on 10% SDS-PAGE and transferred to 0.45-μm nitrocellulose membranes (Hybond A; Amersham, Herts, United Kingdom), which had been blocked with 5% Marvel in Tris-buffered saline (pH 7.5) at 4°C overnight. The primary antibodies to 20S and $E2_{14k}$ were used at a 1:1000 dilution, whereas those for p42 and myosin were used at 1:120 and 1:200, respectively. The secondary antibodies were used at a 1:2000 dilution. Incubation was for 70 min at room temperature, and development was by enhanced chemiluminescence (Amersham). Blots were scanned by a densitometer to

quantitate differences, and a parallel gel was silver stained to ensure equal loading.

Statistical Analysis. Values are given as mean ± SE. Differences between groups were determined by a two-factor ANOVA, followed by Tuckey's test.

RESULTS

The effect of EPA on body weight of mice bearing the cachexia-inducing MAC16 tumor is shown in Fig. 1. Animals with a 8.1% weight loss were randomized to receive either olive oil (control) or EPA p.o. at two dose levels (0.5 and 2.5 g/kg) over a 48-h period. This was considered to be the earliest point at which an effect could be seen. Previous studies (13) used daily dosing over a 10-day period. There was a dose-related reduction in body weight loss in animals treated with EPA, which was significant at both dose levels and an increase in soleus muscle wet weight at 2.5 g/kg EPA (0.13 ± 0.02 and 0.11 ± 0.02 for EPA and control group, respectively).

The role of lysosomal proteolysis in protein degradation in soleus muscle of control mice and those bearing the MAC16 tumor and the effect of EPA on this process is shown in Table 1. In all of the experiments except for that shown in Table 1, there was a significant increase in protein catabolism in soleus muscles from mice bearing the MAC16 tumor (Table 2 and Table 3). However, the overall average from muscles incubated with methylamine ($n = 20$) show control 422 ± 34 μmol of tyrosine g^{-1} 2 h^{-1} and MAC16 695 ± 3 μmol of tyrosine g^{-1} 2 h^{-1} ($P = 0.001$). Lysosomal function in animals bearing the MAC16 tumor was blocked with methylamine, an inhibitor of lysosomal acidification (23). As reported previously (20) in control animals, lysosomal proteolysis did not contribute to overall protein degradation. There was a significant attenuation in overall protein catabolism in both control and tumor-bearing animals administered EPA, and this difference was maintained in the presence of methylamine (Table 1), showing that EPA inhibited a proteolytic pathway other than the lysosomal system.

To determine the role of the Ca^{2+}-dependent pathway, soleus muscles were incubated in the presence of E64, which is known to block calpains I and II (24) in the absence of Ca^{2+}. There was a significant reduction in the rate of protein degradation in both control animals and in mice bearing the MAC16 tumor, suggesting that the Ca^{2+}-dependent pathway contributed to protein degradation (Table 2). EPA significantly inhibited protein degradation in the animals bearing the MAC16 tumor, but this inhibition was not seen in the presence of E64 (Table 2), although E64 produced a significant
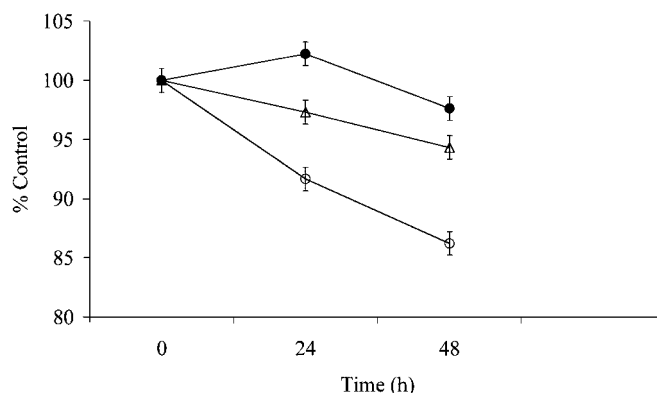

Fig. 1. Effect of p.o. dosing of female NMRI mice bearing the MAC16 tumor and with established weight loss (8.1%) with either olive oil (2.5 g/kg; ○), EPA (2.5 g/kg; ●), or EPA (0.5 g/kg) together with olive oil (2.0 g/kg; △) on body weight (including the tumor mass). Animals were treated at times 0, 24, and 48 h and were terminated 2 h after the final dose for additional studies. Differences from the control group are indicated as: (*a*) $P < 0.05$; (*b*) $P < 0.01$; and (*c*) $P < 0.001$ as determined by one-way ANOVA with Bonferroni correction.

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Table 1 *The effect of inhibition of lysosomal function on protein degradation in soleus muscle of control mice and those bearing the MAC16 tumor, with or without administration of EPA (2.5 g/kg)*

	Protein degradation (μmol tyrosine g^{-1} 2 h^{-1})					
Addition	C[a]	C + EPA	P[b]	T[a]	T + EPA	P[b]
None	533 ± 55	423 ± 36	<0.01	576 ± 24	400 ± 28	<0.05
Methylamine	521 ± 70	451 ± 44	<0.01	423 ± 20	357 ± 7	<0.01
P[b]	NS	NS		<0.05	NS	

[a] C, control mice; T, mice bearing the MAC16 tumor.
[b] NS, not significant; number of animals; C, n = 6; T, n = 8. Control and tumor-bearing groups were run concurrently.

Table 2 *The effect of inhibition of the Ca^{2+}-dependent proteolytic system on protein degradation in soleus muscle of control mice and those bearing the MAC16 tumor, with or without administration of EPA (2.5 g/kg)*

	Protein degradation (μmol tyrosine g^{-1} 2 h^{-1})					
Addition	C[a]	C + EPA	P[b]	T[a]	T + EPA	P[b]
CaCl$_2$	459 ± 4	410 ± 5	NS	618 ± 22	516 ± 12	<0.005
No Ca^{2+} + Inhibitor	384 ± 2	381 ± 21	NS	441 ± 6	475 ± 7	NS
P[b]	<0.01	NS		<0.005	0.05	

[a] C, control mice; T, mice bearing the MAC16 tumor.
[b] NS, not significant; number of animals; C, n = 6; T, n = 8. Control and tumor-bearing groups were run concurrently.

Table 3 *The effect of inhibition of ATP production on protein degradation in soleus muscle of control mice and those bearing the MAC16 tumor, with or without administration of EPA (2.5 g/kg)*

	Protein degradation (μmol tyrosine g^{-1} 2 h^{-1})					
Addition	C[a]	C + EPA	P[b]	T[a]	T + EPA	P[b]
None	476 ± 19	441 ± 25	NS	591 ± 20	440 ± 36	<0.01
−ATP	446 ± 39	436 ± 51	NS	436 ± 23	414 ± 11	NS
P[b]	NS	NS		<0.01	NS	

[a] C, control mice; T, mice bearing the MAC16 tumor.
[b] NS, not significant; number of animals; C, n = 6; T, n = 8. Control and tumor-bearing groups were run concurrently.

reduction in protein degradation in MAC16-bearing mice, with or without EPA. This suggests that EPA had no effect on Ca^{2+}-dependent proteolysis or that it is not completely attenuating this pathway.

To investigate the role of the ATP-dependent pathway, soleus muscles were depleted of ATP after blocking both lysosomal and Ca^{2+}-dependent proteolytic pathways. Under these conditions, protein degradation in nontumor-bearing animals was not significantly reduced, showing that ATP-dependent proteolysis was not important in protein degradation (Table 3). In animals bearing the MAC16 tumor, protein degradation was significantly elevated (P < 0.05), and this was attenuated under conditions of ATP depletion (Table 3), suggesting that this pathway was an important contributor to the excess proteolytic rate, as observed previously (20). EPA caused a significant reduction in the rate of protein degradation in the muscle of tumor-bearing mice, but this was not further reduced in the absence of ATP. This suggests that inhibition of ATP-dependent proteolysis was responsible for the attenuation of overall protein degradation by EPA.

To investigate this possibility, the activity of the proteasome enzyme system was determined in cachectic mice bearing the MAC16 tumor. Previous studies (20) have suggested activation of the ATP-ubiquitin-dependent proteolytic pathway in skeletal muscle from such animals. To confirm this, the "chymotrypsin-like" activity of the proteasome, which is the most dominant catalytic activity, was measured using the fluorogenic substrate SucLLVY-MCA in gastrocnemius muscle and heart of mice bearing the MAC16 tumor (Fig. 2A). In gastrocnemius muscle, enzyme activity increased with increasing weight loss up to 10%, with additional increases in weight loss

causing a decrease in activity, although this was still significantly greater than that found in nontumor-bearing control animals. In contrast, the chymotriptic activity of heart muscle was found to be decreased with weight loss. These results correlate with the loss of gastrocnemius muscle and preservation of heart muscle in cachectic mice bearing the MAC16 tumor. To measure the functional activity of the proteasome after administration of EPA, the "chymotrypsin-like" enzyme activity in gastrocnemius muscle was determined in the absence or presence of the proteasome inhibitor lactacystin (Fig. 2B). There was complete loss of lactacystin-suppressible enzyme activity at both dose levels of EPA, suggesting complete inhibition of the increased proteasome activity seen in skeletal muscle of cachectic animals. To determine cellular expression of proteasome subunits, supernatants of gastrocnemius muscles were Western blotted using MCP231 antibody, a murine monoclonal to the 20S proteasome, which reacts with the six different α-type subunits (Fig. 3). Three
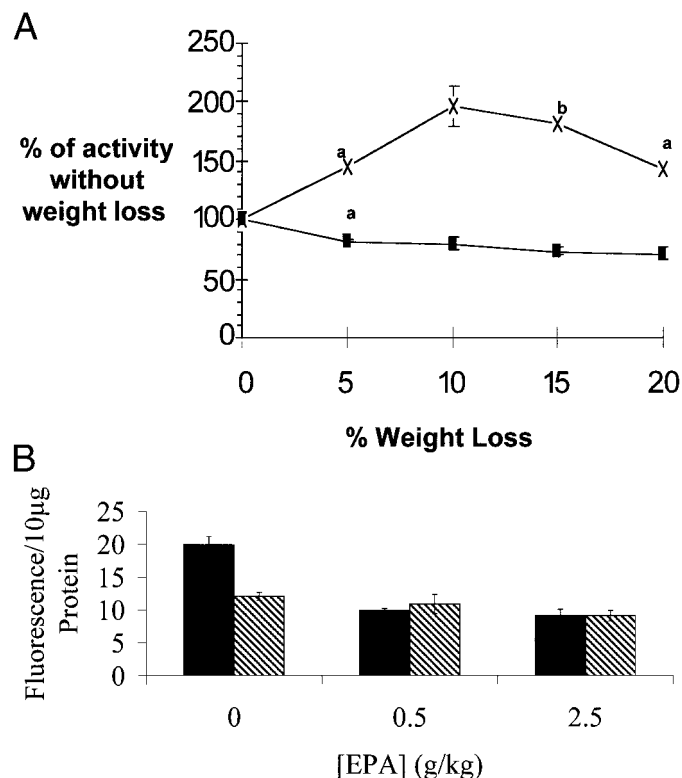

Fig. 2. *A*, the effect of progressive weight loss in mice bearing the MAC16 tumor on the "chymotrypsin-like" enzyme activity of the proteasome in gastrocnemius muscle (*X*) and heart (■). Results are expressed as mean ± SE where n = 9. Differences from nontumor-bearing animals without weight loss are expressed as: (*a*) P < 0.05; and (*b*) P < 0.01 as determined by Student's *t* test. *B*, "chymotrypsin-like" enzyme activity in gastrocnemius muscles from mice treated with EPA, as described in the legend to Fig. 1, in either the absence (■) or presence (▨) of 10 μM lactacystin. Lactacystin caused a significant suppression (P = 0.0015) of the total "chymotrypsin-like" enzyme activity, but there was no significant difference between the values from EPA-treated animals in the presence or absence of 10 μM lactacystin.
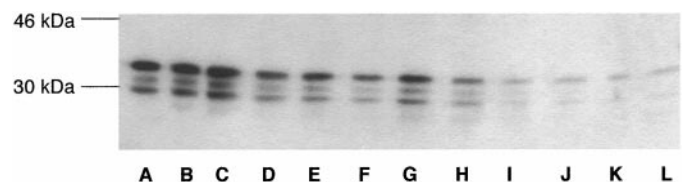

Fig. 3. The effect of EPA on expression of 20S proteasome α-subunits in gastrocnemius muscle from mice bearing the MAC16 tumor. Western blots of extracts from mice treated with olive oil (*Lanes A–D*), EPA, 0.5 g/kg (*Lanes E–H*), or EPA, 2.5 g/kg (*Lanes I–L*). Detection was by MCP231 antibody as described in "Materials and Methods."

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bands were detected at approximately M_r 29,000, 32,000, and 35,000. There was a dose-related decrease in the expression of the 20S proteasome α-type subunits from animals treated with EPA, which was confirmed by densitometric analysis and shown to be 58% ($P < 0.001$ from control) at 0.5 g/kg EPA and 95% ($P < 0.001$ from control) at 2.5 g/kg EPA for the most intense band at M_r 35,000. Other α-type subunits were also reduced appropriately. Equal protein loading was confirmed by silver staining of separate gels. The effect of EPA treatment on expression of p42, an ATPase subunit of the 19S regulator that promotes ATP-dependent association of the 20S proteasome with the 19S regulator to form the 26S proteasome (26) in gastrocnemius muscle, is shown in Fig. 4. EPA caused a dose-related suppression of expression, similar to that found with the 20S proteasome, with a 69% reduction in animals treated with 0.5 g/kg EPA ($P < 0.01$ from control) and a 78% reduction ($P < 0.01$ from control) in animals administered 2.5 g/kg EPA. These results suggest that EPA induces an attenuation of the up-regulation of the expression of proteasome subunits seen in skeletal muscle from cachectic animals. To determine the effect of EPA on other components of the ubiquitin-proteasome pathway, the effect on the expression of the M_r 14,000 ubiquitin-conjugating enzyme ($E2_{14k}$), suggested as being the rate-limiting step in ubiquitin conjugation (27), was determined. Despite changes in proteasome expression, there was no significant change in $E2_{14k}$ expression in gastrocnemius muscle of EPA-treated mice (Fig. 5). To determine whether the reduction in proteasome expression induced by EPA was reflected in muscle protein content, gastrocnemius muscles were also Western blotted for myosin, a major contractile protein. There was an increase in myosin expression with increasing dose levels of EPA (Fig. 6), which was confirmed by densitometric analysis to be 42% (not significant from control) at 0.5 g/kg EPA and 97% ($P < 0.01$ from control) at 2.5 g/kg EPA.

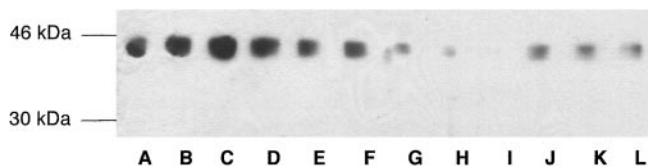As reported previously (13), EPA not only inhibits cachexia in the

Fig. 4. The effect of EPA on p42 expression in gastrocnemius muscle from mice bearing the MAC16 tumor. Western blot of extracts from mice treated with olive oil (*Lanes A–D*), EPA, 0.5 g/kg (*Lanes E–H*), or EPA, 2.5 g/kg (*Lanes I–L*). Detection was by p42 monoclonal antibody as described in "Material and Methods."
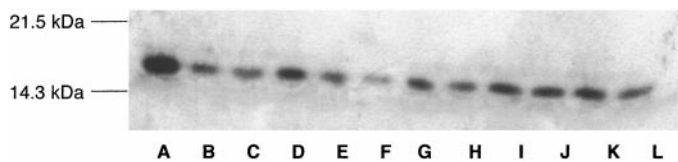

Fig. 5. The effect of EPA on $E2_{14k}$ expression in gastrocnemius muscle from mice bearing the MAC16 tumor. Western blot of extracts from mice treated with olive oil (*Lanes A–D*), EPA, 0.5 g/kg (*Lanes E–H*), or EPA, 2.5 g/kg (*Lanes I–L*). Detection was by rabbit polyclonal antisera to $E2_{14k}$ as described in "Material and Methods."
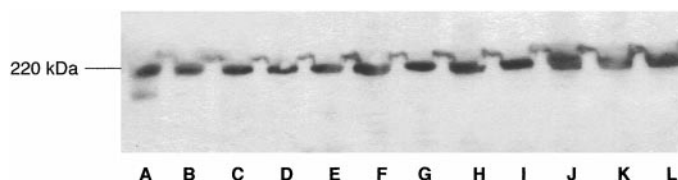

Fig. 6. The effect of EPA on myosin expression in gastrocnemius muscle from mice bearing the MAC16 tumor. Western blot of extracts from mice treated with olive oil (*Lanes A–D*), EPA, 0.5 g/kg (*Lanes E–H*), or EPA, 2.5 g/kg (*Lanes I–L*).
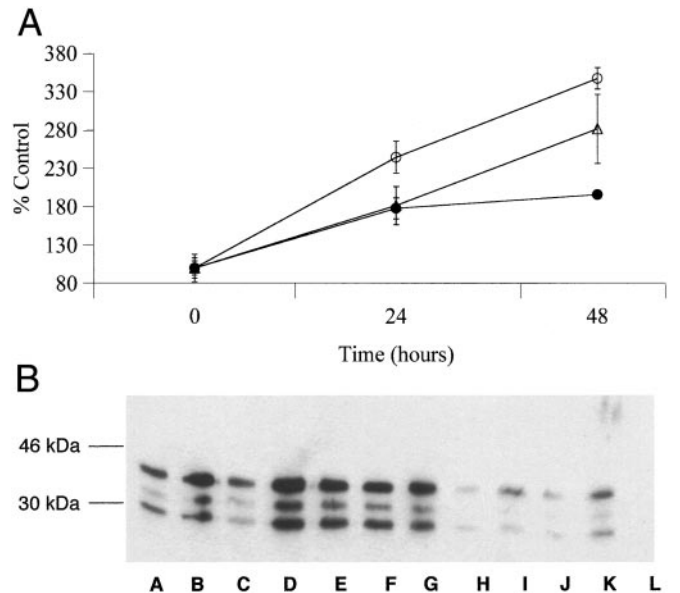
Fig. 7. *A*, effect of p.o. dosing of female NMRI mice bearing the MAC16 tumor with either olive oil (2.5 g/kg; ○), EPA, (2.5 g/kg; ●), or EPA (0.5 g/kg) together with olive oil (2.0 g/kg; △) as described in the legend to Fig. 1. Tumor dimensions were measured by means of calipers and the volume was calculated from the formula:

$$\frac{length \times (width)^2}{2}$$

Differences from the control group are indicated as: (*a*) $P < 0.05$; (*b*) $P < 0.01$; and (*c*) $P < 0.001$ as determined by one-way ANOVA with Bonferroni correction. *B*, the effect of EPA on expression of 20S proteasome α-subunits in the MAC16 tumor. Western blot of extracts from mice treated with olive oil (*Lanes A–D*), EPA, 0.5 g/kg (*Lanes E–H*), or EPA, 2.5 g/kg (*Lanes I–L*). Detection was by MCP231 antibody as described in "Material and Methods."

MAC16 model but also inhibits tumor growth. In the present study, significant tumor stasis was seen at both doses of EPA (Fig. 7A). Because the proteasome has been suggested to be a molecular target for cancer therapy (28), the effect of EPA on the expression of the 20S proteasome in the MAC16 tumor was determined (Fig. 7B). The effect mirrored that on tumor growth with minimal suppression at 0.5 g/kg EPA (19% not significant) but 77% inhibition ($P = 0.009$ from control) at 2.5 g/kg EPA. There was no effect of EPA on the expression of p42 or $E2_{14k}$. Thus, EPA may act as a tumor growth inhibitor by suppression of the expression of the 20S proteasome.

DISCUSSION

Protein catabolism in skeletal muscle could occur via either the lysosomal, calcium-dependent, or the ubiquitin-proteasome-dependent proteolytic pathways. However, it has become apparent from a number of studies that the first two pathways play a minor role in this tissue, contributing less than 15–20% of total protein breakdown, and are not responsible for the catabolism of myofibrillar proteins (29). Instead, the pathway responsible for the accelerated proteolysis induced by starvation (30), sepsis (31), metabolic acidosis (32), denervation atrophy (33), and cachexia induced in rats by either the Yoshida ascites hepatoma (34) or sarcoma (35) and in mice by the MAC16 tumor (20) is attributable to the ubiquitin-proteasome system, although in the latter model the lysosomal and calcium-dependent pathways also play a role. The importance of the proteasome pathway in cancer-induced muscle catabolism in cancer patients has recently been demonstrated (36) by a 2–4-fold increase in tissue levels of mRNA for ubiquitin and 20S proteasome subunits in rectus abdominus muscle of cancer patients compared with patients with benign diseases. In the present study, using *in vitro* preparations of muscle

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excised from mice bearing the MAC16 tumor, it also appears that an ATP-dependent nonlysosomal pathway plays a major role. This pathway is the ubiquitin-proteasome pathway, because we have shown previously increased levels of ubiquitin-conjugated proteins and increased expression of mRNA for the M_r 14,000 ubiquitin carrier protein E2, as well as the C9 proteasome subunit, in gastrocnemius muscle from cachectic mice bearing the MAC16 tumor (20). Increased gene expression of proteasomal subunits has been suggested to be crucial for enhanced protein catabolism in skeletal muscles of rats bearing the Yoshida sarcoma (34).

The eukaryotic proteasome is a multicatalytic protease characterized by three activities against short synthetic peptides. These are hydrophobic (chymotrypsin-like activity), basic (trypsin-like activity), and acidic (peptidyl-glutamyl peptide bond-hydrolyzing activity). Because "chymotrypsin-like" activity represents the most dominant catalytic activity of the proteasome, this activity was used as a measure of functional proteasome activity. Using this method, the proteasome activity of gastrocnemius muscle was increased, whereas heart muscle was slightly decreased correlating with the observed changes in organ weights in cachectic animals.

Previous studies (13) have demonstrated the ability of EPA to down-regulate the increased protein degradation in skeletal muscle of mice bearing the MAC16 tumor. In mice transplanted with the Lewis lung carcinoma transfected with cDNA interleukin-6, which induced cachexia-like symptoms, ubiquitination of muscle proteins is found to be increased compared with that found in nontumor-bearing mice. This elevation was down-regulated after treatment of the animals with either EPA or docosahexaenoic acid, which were also shown to attenuate the development of cachexia in this model (37). However, ubiquitin has roles other than in proteolysis (38) and, therefore, more direct evidence is required before this is interpreted as an effect on the ATP-ubiquitin-dependent proteolytic pathway.

Using isolated gastrocnemius muscles from mice bearing the MAC16 tumor in the presence of inhibitors, there appeared to be no effect of EPA on either lysosomal or calcium-dependent proteolysis, but ATP-dependent proteolysis was completely inhibited. *In vivo* studies using mice transplanted with the MAC16 tumor and administered EPA showed that functional proteasome activity in gastrocnemius muscle was also significantly decreased, as was the expression of 20S proteasome immunoreactive protein and the p42 regulator, whereas myosin expression was increased. These results suggest that EPA directly affected the expression of proteasome subunits rather than acting as a direct inhibitor of proteasome activity.

The mechanism by which EPA attenuates proteasome expression is not known but may be related to the ability to suppress 15-hydroxyeicosatetraenoic acid production, which correlated with inhibition of PIF-induced muscle catabolism in a surrogate model system (39). 15-hydroxyeicosatetraenoic acid also produced a significant increase in protein degradation in this system.

The effect of EPA on proteasome expression may also explain the growth inhibitory effect of this fatty acid on certain tumors. In the MAC16 tumor, inhibition of the expression of the 20S proteasome by EPA correlated with growth inhibition. Other studies (40) have shown that treatment of the pancreatic cancer cell line MIA Pa Ca-2 with EPA caused cell cycle arrest in G_2-M and the induction of apoptosis. Accumulation of cells in the G_2-M phase of the cell cycle and subsequent apoptosis is also induced in a prostate cancer cell line by PS-341, a dipeptide boronic acid analogue that inhibits the chymotryptic activity of the proteasome (41).

These results provide a basis on which to understand the complex biochemical processes involved in the use of EPA in the treatment of cancer cachexia. Additional studies are required to fully understand how EPA modulates the activity of the proteasome pathway.

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