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Downregulation of ubiquitin-dependent protein degradation in murine myotubes during hyperthermia by eicosapentaenoic acid

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Abstract

Muscle atrophy in a number of acute wasting conditions is associated with an increased activity and expression of the ubiquitin–proteasome proteolytic pathway. Although different initiators are involved, it is possible that the intracellular signalling events leading to upregulation of this pathway are the same in all catabolic conditions. This study investigates hyperthermia in murine myotubes as a model for increased protein degradation through the ubiquitin–proteasome pathway. The effect of eicosapentaenoic acid (EPA) on this process should identify common elements, since EPA has been shown to attenuate induction of the ubiquitin–proteasome pathway in cancer cachexia. Increasing the temperature of myotubes caused a progressive increase in protein degradation. This was associated with an increased proteasome 'chymotrypsin-like' enzyme activity, as well as increased expression of both mRNA and protein for 20S proteasome subunits and the ubiquitin-conjugating enzyme ($E2_{14k}$). This upregulation was not seen in cultures treated with EPA (50 μM), suggesting that it acts to prevent transcriptional activation of the ubiquitin–proteasome pathway in hyperthermia. These results suggest that protein catabolism in hyperthermia and cancer cachexia is mediated through a common pathway.

Keywords: Ubiquitin–proteasome proteolysis; Hyperthermia; EPA; Transcriptional activation

Wasting of skeletal muscle is a common metabolic response to several disease states including cancer, sepsis, severe injury, burns, and renal failure. Loss of skeletal muscle results in weakness and immobility, delaying or preventing rehabilitation, while continual erosion of respiratory muscles eventually results in the death of the patients through hypostatic pneumonia [1]. Although reduced protein synthesis and inhibited uptake of amino acids contribute to the atrophy of skeletal muscle, increased protein degradation, and in particular breakdown of the myofibrillar proteins actin and myosin, are probably the most important contributory factors. There are three main proteolytic pathways that could contribute to muscle loss in cachexia, lysosomal, calcium-activated proteases (calpains) and the ubiquitin–proteasome proteolytic pathway [2]. It has been estimated that the former two processes contribute less than 15–20% of total protein breakdown in muscle, although calpains appear to be required for the release of myofilaments by digestion of the Z-discs in the muscle sarcomere [3]. Lysosomes do not breakdown myofibrillar proteins [4], and the ubiquitin–proteasome proteolytic pathway is considered to play the major role in intracellular protein degradation [2]. In this process, ubiquitin becomes activated and attached to the protein substrate and the polyubiquitinated protein is recognized for degradation by the 26S proteasome complex [2].

Glucocorticoids have been considered to be important mediators of the activation of the ubiquitin–proteasome proteolytic pathway in sepsis [5], while in metabolic

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acidosis it may arise indirectly through the release of cytokines from macrophages [6]. In cancer cachexia a tumour factor, proteolysis-inducing factor (PIF) is responsible for upregulation of this pathway [7]. Although different extracellular signals are involved, it is possible that the intracellular signalling events leading to increased gene transcription of proteasome subunits are the same in all catabolic conditions. PIF-induced protein degradation is attenuated by the polyunsaturated fatty acid eicosapentaenoic acid (EPA) through inhibition of 15-hydroxyeicosatetraenoic acid (15-HETE) formation, via 15-lipoxygenase (15-LOX) [8]. EPA was shown to effectively down-regulate proteasome expression in a murine model of cancer cachexia [9]. In addition, both EPA and a 15-LOX inhibitor, CV-6504, also down-regulated increased protein breakdown and expression of key regulatory components of the ubiquitin–proteasome pathway in acute fasting in mice [10], suggesting that the signalling pathway was the same as in cancer cachexia.

The purpose of the present study was to determine whether the same signalling pathway, which is inhibited by EPA, operates in other models of muscle catabolism. This study investigates hyperthermia using C_2C_{12} myotubes as a model of skeletal muscle. An elevated temperature that accompanies sepsis and severe infections may contribute to muscle proteolysis [11], and hyperthermia has been shown to stimulate muscle protein breakdown through the ubiquitin–proteasome pathway [12].

Materials and methods

Materials. L-[2,6-^{3}H]Phenylalanine (sp. act. 2.00 TBq/mmol) was purchased from Amersham Biosciences (Amersham, Berks, UK). Foetal calf serum (FCS), horse serum (HS), and Dulbecco's modified Eagle's medium were purchased from Life Technologies (Paisley, UK). Mouse monoclonal antibody to 20S proteasome α-subunits was from Affiniti Research Products (Exeter, UK). Rabbit polyclonal antisera to ubiquitin-conjugating enzyme ($E2_{14k}$) were a gift from Dr. Simon Wing, McGill University, Montreal, Canada. Rabbit polyclonal antisera to mouse actin were from Sigma–Aldridge (Dorset, UK) as was EPA (99%) as the free acid. Peroxidase-conjugated goat anti-rabbit antibody and peroxidase-conjugated rabbit anti-mouse antibody were purchased from Dako (Cambridge, UK).

Cell culture. C_2C_{12} myoblasts were grown in DMEM supplemented with 10% FCS. When the myoblasts became confluent, they were allowed to differentiate into myotubes by replacing the medium with DMEM containing 2% HS. Medium was changed every 2 days and differentiation was complete within 5–7 days.

Measurement of protein degradation. Myotubes were pre-labelled with L-[2,6-^{3}H]phenylalanine as described [13] for a 24 h period, after which they were washed extensively in PBS, and incubated in fresh DMEM for a 2 h period at 37 °C, until no more radioactivity appeared in the medium. Protein degradation was measured by the release of [2,6-^{3}H]phenylalanine into the medium after 6 h incubation at 37, 39, 41 or 43 °C in the presence of 2 mM 'cold' phenylalanine to prevent re-incorporation of the radio-label. Cultures were incubated with or without a 2 h pre-treatment with 50 μM EPA, a concentration which has previously been shown to be effective in attenuating increased protein degradation [8].

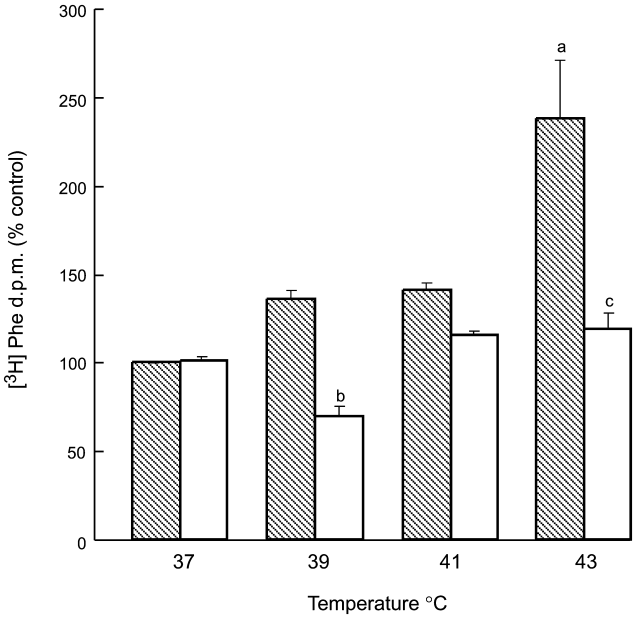

Fig. 1. The effect of temperature with (□) and without (▨) EPA (50 μM) on total protein degradation in C_2C_{12} myotubes over a 6 h period. Myotubes were incubated at the indicated temperatures and total protein degradation was determined as described in methods. The experiment was repeated three times. Differences from control are indicated as a, $p < 0.005$, while differences in the presence of EPA are indicated as b, $p < 0.01$ or c, $p < 0.005$.

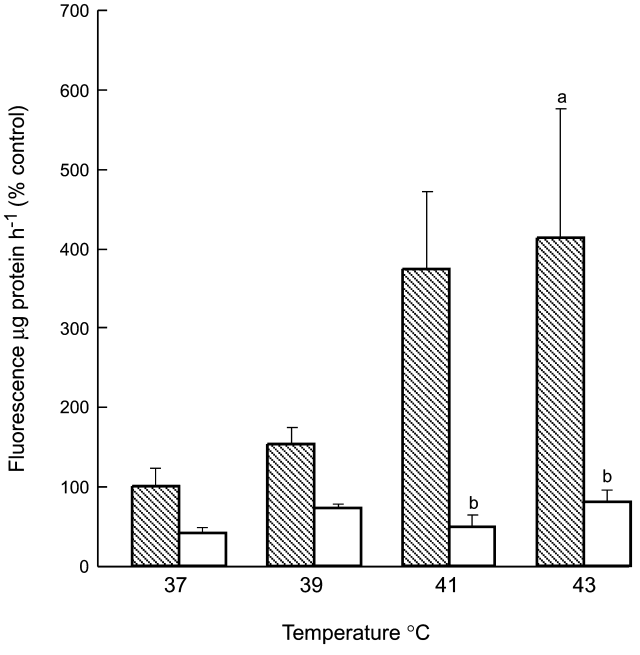

Fig. 2. The effect of temperature with (□) and without (▨) EPA (50 μM) on proteasome chymotrypsin-like enzyme activity in C_2C_{12} myotubes over a 6 h period. Myotubes were incubated at the indicated temperatures, and enzyme activity was determined as described in methods. The experiment was repeated three times. Differences from control are indicated as a, $p < 0.05$, while differences in the presence of EPA are indicated as b, $p < 0.05$.

Measurement of proteasome enzyme activity. Proteasome 'chymotrypsin-like' enzyme activity was measured fluorimetrically by the release of aminomethyl coumarin (AMC) from the fluorogenic peptide succinyl-LLVY-AMC as described [8]. Activity was determined in the absence and presence of the specific proteasome inhibitor lactacystin (10 μM). Only lactacystin-suppressible activity was considered to be proteasome specific. Activity was adjusted for the protein concentration of the sample determined using the Bradford assay.

Western blot analysis. Samples of cytosolic protein (5–10 μg) were resolved on 12% sodium dodecyl sulphate, polyacrylamide gels, and transferred to 0.45 μm nitrocellulose membranes (Hybond A, Amersham, UK), which had been blocked with 5% Marvell in Tris-buffered saline, pH 7.5, at 4 °C overnight. The primary antibodies were used at a dilution of 1:1500, except actin (1:200), while the secondary antibodies were used at a dilution of 1:1000. Incubation was performed for 1 h at room temperature and development was effected by enhanced chemiluminescence. Blots were stripped and probed for total cellular actin as a loading control.

Quantitative competitive RT-PCR. Total RNA was extracted from myotubes using TR1 reagent (Sigma–Aldridge, Dorset, UK) and the concentration was determined from the absorbance at 260 nm. To quantitate mRNA increasing amounts of competitor RNA, which differed from the mRNA of interest by containing a short deletion, were added to 250 ng of total RNA, and coamplified using RT-PCR as described [14]. The amount of specific mRNA was calculated from the amount of competitor when equal amounts of PCR product were obtained from the competitor and target RNA.

Statistical analysis. Results are expressed as means ± SEM. Differences were determined using two-way ANOVA followed by Tukey–Kramer multiple comparison test.

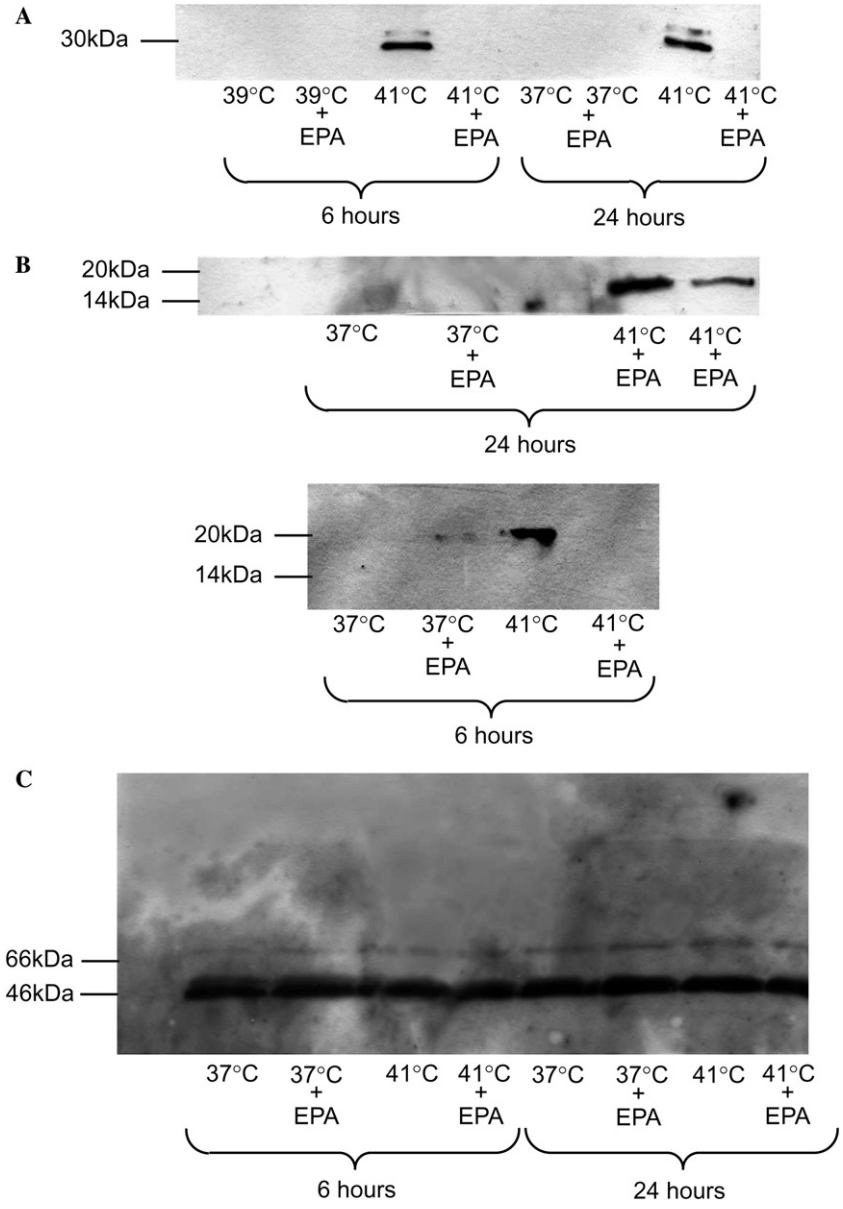

Fig. 3. Western blotting of cytosolic extracts of murine myotubes for 20S proteasome α-subunit (A), $E2_{14k}$ (B), and actin loading control (C). Myotubes were heated at 39 or 41 °C for 6 and 24 h, in the absence or presence of EPA, as indicated. The blots shown are representative of three separate experiments.

Results

The effect of temperature on total protein degradation in C_2C_{12} myotubes is shown in Fig. 1. There was no significant effect between 37 and 41 °C, but a 2–5-fold increase in degradation over a 6 h period at 43 °C. There was no increase in protein degradation at 43 °C, over myotubes maintained at 37 °C, when the cells were pre-treated for 2 h with 50 μM EPA (Fig. 1).

Although protein degradation was not significantly increased at 41 °C, there was a significant increase (4-fold) in proteasome proteolytic activity, determined as the chymotrypsin-like enzyme activity, the predominant proteolytic activity of the proteasome (Fig. 2). As with protein degradation proteasome proteolytic activity was completely attenuated in cultures pre-treated with 50 μM EPA when incubated at either 41 or 43 °C.

To determine if the increased proteasome proteolytic activity was reflected in increased protein expression, cellular supernatants were subjected to Western blotting using an antibody to the 20S proteasome α-subunits (Fig. 3A). There was an increased expression of proteasome α-subunits in myotubes incubated at 41 °C for both 6 and 24 h, but this was not seen in cultures treated with EPA. There was also an increase in expression of the ubiquitin conjugating enzyme $E2_{14k}$ after 24 h at 41 °C, which again was attenuated by EPA (Fig. 3B). Actin was used as a loading control (Fig. 3C) and shows that equal amounts of cellular protein were added to each lane.

To determine whether the effect was active at the transcriptional or translational level mRNA levels for the C5 proteasome subunit, a β-subunit was determined after 2 and 4 h using quantitative competitive RT-PCR (Fig. 4). There was no effect after 2 h (not shown) but there was a twofold increase in mRNA for C5 proteasome subunit after 4 h in cultures incubated at 41 °C and this effect was completely attenuated by EPA (Fig. 4A). Expression of E2mRNA was also significantly increased at both 2 and 4 h in myotubes heated to 41 °C, but again this was not seen in the presence of EPA (Figs. 4B and C). These results suggest that EPA acts at the transcriptional level to block increased mRNA expression of key components of the ubiquitin–proteasome pathway.

Discussion

The ubiquitin–proteasome pathway appears to be responsible for the bulk of myofibrillar protein degradation in a number of catabolic conditions including cancer cachexia, sepsis, diabetes, immobilization and weightlessness, renal tubular defects, and burn injuries [2]. Although different stimuli are likely to be responsible for the induction of this pathway in the different catabolic states, it is hypothesized that the intracellular signalling pathways leading to transcriptional activation of proteasome subunits, and other major components of the pathway, will be the same. Some evidence for this has been obtained from the ability of EPA to down-regulate protein degradation and the ubiquitin–proteasome pathway during acute starvation in mice [10]. The results of the current study also support a similar signalling pathway being responsible for the induction of the ubiquitin–proteasome pathway in hyperthermia.

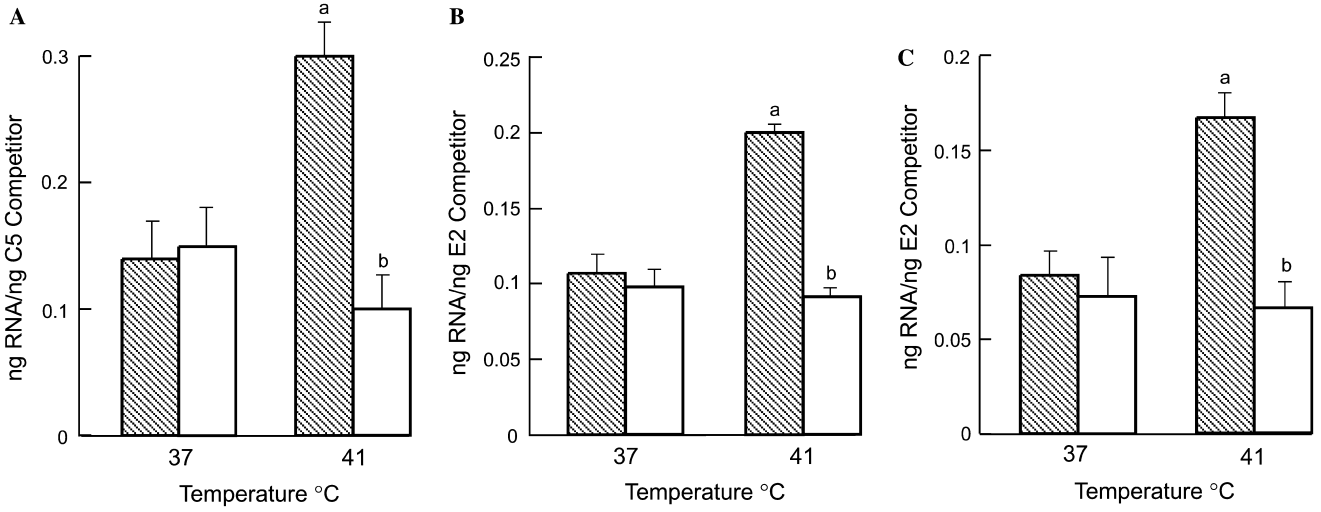

Fig. 4. Effect of temperature on the expression of mRNA for C5 proteasome subunit (A,B) and $E2_{14k}$ (C,D) in the absence (▨) or presence (□) of EPA (50 μM). Expression was measured at both 2 h (A,C) and 4 h (B,D). Differences from control are indicated as a, $p < 0.05$, while differences in the presence of EPA are shown as b, $p < 0.01$. The experiment was repeated three-times.

Although the mechanism for the induction of the ubiquitin–proteasome pathway has not been determined in hyperthermia, there are certain similarities in other cases of muscle wasting, particularly with regards to the role of nuclear transcription factor-κB (NF-κB). Thus, increased nuclear binding of NF-κB to its targeted DNA sequences has been shown to mediate the protein loss and induction of the ubiquitin–proteasome pathway, induced by tumour necrosis factor-α (TNF-α), [15] and PIF [13], as well as reactive oxygen species [16], which are thought to be responsible for muscle atrophy associated with hindlimb unloading, as a model of skeletal muscle disuse [17]. In addition, upregulation of NF-κB activity in extensor digitorum longus muscles was seen within 4 h of the induction of sepsis [18]. If this pathway is common to all models of muscle atrophy, it suggests a potential mechanism by which EPA could attenuate muscle protein degradation mediated through the ubiquitin–proteasome pathway, since in PIF-induced activation of this pathway EPA has been shown to prevent nuclear accumulation of NF-κB, by stabilizing the I-κBα/NF-κB complex, by inhibiting upstream signalling pathways [13]. This suggests that other antagonists of activation of NF-κB may also be effective in attenuating skeletal muscle atrophy. Thus, the leucine metabolite β-hydroxy-β-methylbutyrate, which also inhibits PIF-induced activation of NF-κB and prevents PIF-induced protein degradation [19], has been shown to increase muscle mass and body weight in patients with cancer [20] and AIDS-associated wasting [21]. Also, resveratrol, an inhibitor of the upstream kinase IκB-kinase (IKK), involved in the phosphorylation and subsequent degradation of IκB, has been shown to inhibit weight loss and protein degradation in the skeletal muscle of mice bearing a cachexia-inducing tumour [22].

No matter what the exact mechanism for the induction of the ubiquitin–proteasome pathway in hyperthermia, the present results suggest that EPA, which is already used clinically to prevent loss of muscle mass in cancer cachexia [23], may also be effective in preventing loss of muscle mass in patients with elevated temperatures, as seen in sepsis and severe infection.

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