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The effect of eicosapentaenoic and docosahexaenoic acid on protein synthesis and breakdown in murine C2C12 myotubes

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ABSTRACT

Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) have been found to stimulate protein synthesis with little information regarding their effects on protein breakdown. Furthermore whether there are distinct effects of EPA and DHA remains to be established. The aim of the current study was to determine the distinct effects of EPA and DHA on protein synthesis, protein breakdown and signalling pathways in C2C12 myotubes. Fully differentiated C2C12 cells were incubated for 24 h with 0.1% ethanol (control), 50 μM EPA or 50 μM DHA prior to experimentation. After serum (4 h) and amino acid (1 h) starvation cells were stimulated with 2 mM L-leucine and protein synthesis measured using ^{3}H-labelled phenylalanine. Protein breakdown was measured using ^{3}H-labelled phenylalanine and signalling pathways (Akt, mTOR, p70S6k, 4EBP1, rps6 and FOXO3a) via Western blots. Data revealed that after incubation with EPA protein synthesis was 25% greater ($P < 0.05$) compared to control cells, with no effect of DHA. Protein breakdown was 22% ($P < 0.05$) lower, compared to control cells, after incubation with EPA, with no effect of DHA. Analysis of signalling pathways revealed that both EPA and DHA incubation increased ($P < 0.05$) p70s6k phosphorylation, EPA increased ($P < 0.05$) FOXO3a phosphorylation, with no alteration in other signalling proteins. The current study has demonstrated distinct effects of EPA and DHA on protein metabolism with EPA showing a greater ability to result in skeletal muscle protein accretion.

1. Introduction

Controlling the size of skeletal muscle has great importance in the maintenance of physical function, particularly during conditions such as sarcopenia, which can be described as the gradual decline in skeletal muscle mass and function with age. The underlying mechanisms responsible for this condition are as yet unknown but are proposed to include factors such as altered protein metabolism, motor unit remodelling and chronic low grade inflammation. One clear observation is that ageing skeletal muscle exhibits a retarded increase in protein synthesis in response to anabolic stimuli, such as leucine or resistance exercise, compared to young muscle [1–3]. Furthermore, a reduction in the effectiveness of insulin to limit muscle protein breakdown has been observed in ageing skeletal muscle [4]. These factors result in an imbalance in protein turnover and thereby a reduction in skeletal muscle mass in older adults.

One factor known to influence the maintenance of muscle mass is nutrition and in that regard, the two long chain *n*-3 polyunsaturated fatty acids (LCn-3PUFA), Eicosapentaenoic acid (EPA) and Docosahexaenoic acid (DHA), found in oily fish and fish oil have been demonstrated to have anabolic effects in both animal and human models. Gingras et al. (2007) reported that fish oil feeding (menhaden oil containing 13.5% EPA and 14.4% DHA) increased whole body protein anabolism, concurrent with an increase in insulin sensitivity and enhanced activity of the Akt-mTOR-p70s6k anabolic signalling pathway in young steers [5]. Similarly, Smith et al. (2011) found that eight-weeks LCn-3PUFA supplementation (4 g Lovaza containing 1.86 g EPA and 1.5 g DHA, per day) enhanced muscle protein synthesis during a hyperinsulinemic-hyperaminoacidemic clamp in older people [6]. This effect was associated with an increase in the phosphorylation of p70s6k with no alterations in plasma levels c-reactive protein (CRP), tumour necrosis factor-α (TNF-α) or interleukin-6 (IL-6). Furthermore in a recent study from our lab we have shown that eight-weeks fish oil supplementation (EPAX 6000 containing 49.6% EPA and 50.4% DHA) improved whole body glucose turnover, enhanced anabolic signalling (e.g. p70s6k) and tended to preserve total lean mass [7].

In each of the aforementioned studies EPA and DHA were delivered in roughly equal quantities and to ultimately optimise the effects of supplementation knowledge of the distinct effects of EPA and DHA on protein anabolism and catabolism in skeletal muscle is needed. Palmer and Wahle (1987) reported that in fasted rabbits individually arachidonic acid, EPA and DHA (0.2 and 1 μM) had no

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effect on the rate of isolated muscle protein synthesis or degradation [8]. In studies looking at the effects of EPA alone individual pre-incubation with 50 μM EPA, in C2C12 myoblasts, has been reported to reduce the effect of proteolysis inducing factor (PIF) on protein degradation, with a small increase in protein synthesis independent of PIF exposure [9]. Similar effects have been found in tumour bearing mice and in arthritic rats [10,11]. It has also been reported that 50 μM EPA can attenuate the deleterious effects of TNF-α on skeletal muscle C2C12 differentiation and inhibit TNF-α induced apoptosis via a reduced caspase-8 activity in C2C12 myotubes [12]. Furthermore it has been shown that the ubiquitin proteasome system, which may account for up to 80% of proteolysis during muscle wasting [13], can be down regulated by EPA [10]. Collectively, therefore, individual EPA treatment appears to suppress catabolic stimuli induced protein breakdown in skeletal muscle in both *in vitro* and *in vivo*, with little information on their effects on muscle protein synthesis, particularly after metabolic stimuli such as amino acids. Therefore, further study is needed to compare the effects of EPA and DHA on skeletal muscle anabolism and catabolism.

The aim of the present study is to determine the distinct effects of EPA and DHA on protein synthesis, anabolic signalling pathways and protein breakdown. We hypothesized that EPA would be more potent in enhancing L-leucine stimulated protein synthesis when compared to DHA. In addition, we hypothesized that EPA may reduce protein breakdown, to a great extent than that with DHA, in C2C12 myotubes.

2. Materials and methods

2.1. C2C12 cell culture

Murine C2C12 cells were cultured in 10 ml growth medium containing 88% high-glucose Dulbecco's Modified Eagles's medium (DMEM) (Sigma, St. Louis, USA), 10% foetal calf serum (FCS) and 2% glutamine (Thermo Fisher, Waltham, MA, USA), known as proliferation medium (PM) in T75 cm^2 flasks at 37 °C and 5% CO_2 for 48–72 h until cell proliferation met 50–70% confluence, at which point cells were passaged or used for experiments. For all experiments, 2×10^6 cells were seeded and grown in plastic 6-well plates, in 2 ml PM. When 100% confluence was reached myoblasts were induced to fuse and form myotubes by switching the media to 2 ml differentiation medium (DM) containing 96% high-glucose DMEM, 2% horse serum (HS) and 2% glutamine. DM was changed every 24 h for 5–6 days, at which point cells were used for experiments.

3. Outcome measurements

3.1. Protein synthesis measurement

EPA and DHA were purchased from Cayman Chemical (MI, USA). Fifty millimolar EPA and DHA stock solutions were prepared by solubilising in 100% ethanol and aliquoted into a light resistant glass vials and stored at −20 °C. EPA, DHA and control (ethanol) treatments were prepared on the treatment day in pre-warmed DM containing 4% bovine serum albumin (BSA) at 37 °C. Briefly, a 1:1000 dilution of either EPA, DHA or absolute ethanol was added into pre-warmed DM containing 4% fatty acid free BSA to make a final concentration as 50 μM for LCn-3PUFA and a final concentration of ethanol of 0.1%. All treatments were incubated in water bath at 37 °C for at least 1 h before being applied to cells.

For treatment, C2C12 myotubes (day 5 or 6) were washed twice with pre-warmed phosphate buffer saline (PBS) (Sigma, St Louis, USA). Then, 2 ml of either control, EPA or DHA treatments were added to each well and incubated in a humidified condition at 37 °C and 5% CO_2 for 24 h. Protein synthesis measurements were obtained with minor modification from a previous protocol described [14]. After 24-h treatment, media were discarded, C2C12 myotubes were washed twice with pre-warmed PBS and cells serum starved in pre-warmed low-glucose DMEM (Sigma, St. Louis, USA) at 37 °C and 5% CO_2 for 4 h. Cells were then washed twice with pre-warmed PBS and amino acids starved in pre-warmed Hanks' Balanced Salt solution (HBSS) (Sigma, St. Louis, USA) at 37 °C and 5% CO_2 for 1 h. After this period 2 mM L-leucine (Sigma, St. Louis, USA) was added and the cells incubated at 37 °C and 5% CO_2 for 30 min. L-leucine stimulation was employed due to previous work showing that it is the most potent amino acid in stimulating protein anabolism [15]. Dilution treatments were discarded and cells were washed twice with pre-warmed PBS. Subsequently, cells were incubated with 1.5 μCi L-[2,6-^{3}H]phenylalanine (American Radiolabeled Chemicals, St. Louis, USA) in pre-warmed HBSS containing 2 mM non-labelled phenylalanine (Sigma, St. Louis, USA) at 37 °C and 5% CO_2 for 1 h.

Incorporation of L-[2,6-^{3}H]phenylalanine into the cells was then measured. Briefly, C2C12 myotubes were washed with ice-cold PBS and 5% Trichloroacetic acid (TCA). Cells were scraped and removed into a microcentrifuge tube. Each well was then washed a further twice with 5% TCA and samples placed on ice for 1 h. Samples were centrifuged at 6000*g* for 10 min, the supernatant discarded and the pellets washed three times with 5% TCA. Each wash was followed by vortexing and centrifugation at 10,000*g* for 10 min. The pellets were finally dissolved in 0.1 M NaOH and 0.1% sodium dodecyl sulfate (SDS). Protein concentration was measured by the Bicinchoninic acid (BCA) assay (Thermo Fisher, Waltham, MA, USA). The protein samples were suspended in Multi-purpose liquid scintillation cocktail (Meridian Biotechnologies, Surrey, UK) and L-[2,6-^{3}H]phenylalanine incorporation was measured with a Wallac 1409 Liquid Scintillation counter (PerkinElmer, Waltham, MA, USA). Protein synthesis was expressed as incorporation of L-[2,6-^{3}H]phenylalanine in disintegrations per minute (DPM) per nanogram of total TCA precipitated protein.

3.2. Protein breakdown measurement

To determine the protein breakdown, C2C12 myotubes were treated with either EPA, DHA or control treatments. Treatments were prepared as described above with the addition of 2 mM non-labelled phenylalanine. Protein breakdown measurement was performed with minor modifications from the protocols described [16]. C2C12 myotubes (day 5 or 6) were pre-labelled with L-[2,6-^{3}H]phenylalanine (10 μCi sp.act 53 Ci $mmol^{-1}$) in pre-warmed DM at 37 °C and 5% CO_2 for 24 h. After 24 h, myotubes were washed twice with pre-warmed PBS and incubated for two hours at 37 °C and 5% CO_2 in DM without phenol red. Myotubes were then treated with EPA, DHA or control. After 24-h treatment 400 μl dilution of media was removed to Multi-purpose liquid scintillation cocktail and the amount of L-[2,6-^{3}H]phenylalanine released quantified with a Wallac 1409 Liquid Scintillation counter (PerkinElmer, Waltham, MA, USA). The remaining cells were washed and protein extracted and measured as described above (protein synthesis section). Protein breakdown was expressed as the amount of L-[2,6-^{3}H]phenylalanine released into the medium in DPM per nanogram protein.

3.3. Signalling pathways

C2C12 myotubes were washed twice with ice-cold PBS and myotubes were scraped on ice in homogenization buffer (containing 50 mM Tris–HCl, 1 mM EDTA, 1 mM EGTA, 1% Triton x-100, 1 in 50 Protease inhibitor cocktail Sigma, 10 mM β-glycerophosphate, 50 mM sodium fluoride, and 1 mM Sodium orthovanadate).

Samples were incubated on ice for 10 min, centrifuged at 13,000*g* for 10 min, supernatant removed and protein concentration measured by BCA assay. Samples were then diluted with3× Laemmlli SDS sample buffer (containing 30% glycerol, 0.625 M Tris (pH 6.8), 20% (w/v) SDS, 0.5% (w/v) bromophenol blue, dH_2O, and 1:9 dilution β-mercaptoethanol) and homogenization buffer to give a final working concentration of 1 μg/μl.

Forty to eighty microgram of protein was loaded and separated by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) at 200 v for 50 min (Criterion™ XT Precast Gels from Bio–Rad, Hercules, CA, USA) and transferred to polyvinylidene difluoride membranes (Amersham Hybond™-P, GH Healthcare, NA) by semi-dry transfer gel method at 15 v for 1 h. After blocking membranes for 1 h (5% non-fat skimmed milk in Tris-buffered saline, 0.1% Tween-20) membranes were then incubated with primary antibody overnight at 4 °C. The primary antibodies used were phospho-Akt$^{[Ser473]}$, Akt, phospho-mTOR$^{[Ser2448]}$, mTOR, phospho-eukaryotic initiation factor 4E (eIF4E) binding protein 1 (4EBP1)$^{[Thr37/46]}$, 4EBP1, phospho-p70s6k$^{[Thr389]}$, p70s6k, phospho-rps6$^{[Ser235/236]}$, phospho-FOXO3a$^{[Ser253]}$ and β-actin from Cell Signalling Technology (Danvers, MA, USA). Membranes were then incubated with secondary antibody. Protein bands were identified with Quantity one Fluor-*S*™ MultiImager software version 4.5.1 (Bio–Rad, Hercules, CA, USA) and bands were quantified by using ImageJ 1.42q (NIH, USA).

3.4. Statistical analyses

All data were expressed as means and standard deviation (SD). Prism version 5 software was used to analyse all data. A one-way ANOVA with Bonferroni post hoc tests was carried out to determine the difference in all outcomes between treatment conditions. $P < 0.05$ was considered as a statistical significant difference. Three to five independent experiments per condition were carried out in each outcome.

4. Results

4.1. Protein synthesis measurement

After 24 h treatment of C2C12 myotubes with EPA and DHA the one-way ANOVA revealed a difference in protein synthesis between groups ($P < 0.05$). Post hoc analysis showed that protein synthesis was higher in the EPA treated, compared to control cells ($P < 0.05$). There was no difference in protein synthesis between DHA and control cells (Fig. 1).

4.2. Protein breakdown measurement

In C2C12 myotubes the ANOVA showed a difference in protein breakdown between groups ($P < 0.05$). Post hoc analysis showed that protein breakdown was lower in the EPA treated, compared to control cells ($P < 0.05$). There was no difference in protein breakdown between DHA and control cells (Fig. 2).

4.3. Signalling pathways

In C2C12 myotubes the ANOVA revealed differences between groups in the phosphorylation of p70s6k$^{[Thr389]}$ with post hoc analysis showing that p70s6k$^{[Thr389]}$ phosphorylation was greater in EPA and DHA treated cells, compared to control cells. Furthermore the ANOVA also revealed that EPA treated cells had a greater phosphorylation of FOXO3a$^{[Ser253]}$ compared to control treated cells, with no differences with DHA. There were no differences in the phosphorylation of 4EBP1$^{[Thr37/46]}$ of rps6$^{[S235/236]}$, mTOR$^{[Ser2448]}$ or Akt$^{[Ser473]}$ in C2C12 myotubes between treatments (Fig. 3).

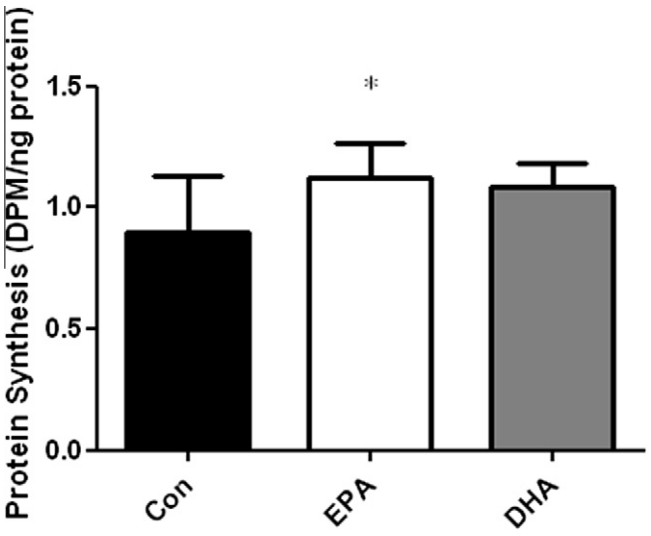

Fig. 1. Protein synthesis in control, EPA and DHA treatments in C2C12 myotubes after L-leucine stimulation. Protein synthesis was expressed as incorporation of L-[2,6-^{3}H]phenylalanine in disintegrations per minute (DPM) per nanogram of total TCA precipitated protein. Data are mean + SD (from 4 independent experiments in duplicate). A one-way ANOVA with Bonferroni post hoc tests was carried out to determine the difference between treatment conditions and $P < 0.05$ was set to detect a significant difference. * Denotes a significant difference from control treatment.

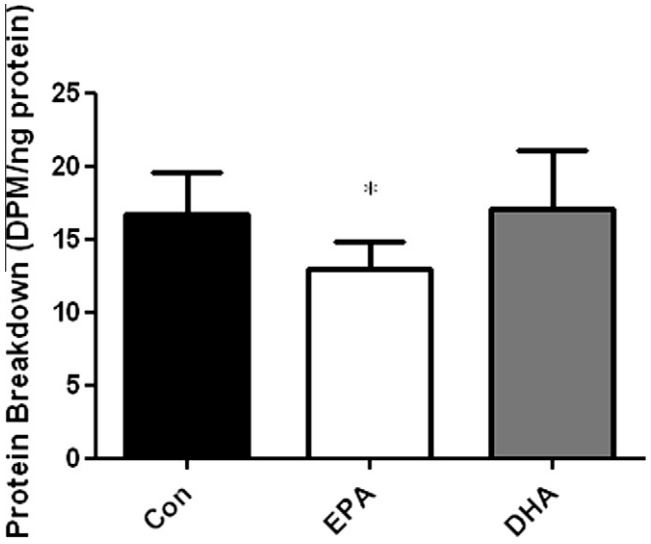

Fig. 2. Protein breakdown in control, EPA and DHA treatments in C2C12 myotubes. Protein breakdown was expressed as the amount of L-[2,6-^{3}H]phenylalanine released into the medium in disintegrations per minute (DPM) per nanogram protein. Data are mean + SD (from 4 independent experiments in duplicate). A one-way ANOVA with Bonferroni post hoc tests was carried out to determine differences between treatment conditions and $P < 0.05$ was set to detect a significant difference. * Denotes a significant difference from control treatment.

5. Discussion

The current study investigated the potential for distinct effects of EPA and DHA on protein synthesis, anabolic signalling pathways and protein breakdown in C2C12 myotubes. We have demonstrated that pre-treatment for 24 h with EPA alone enhanced

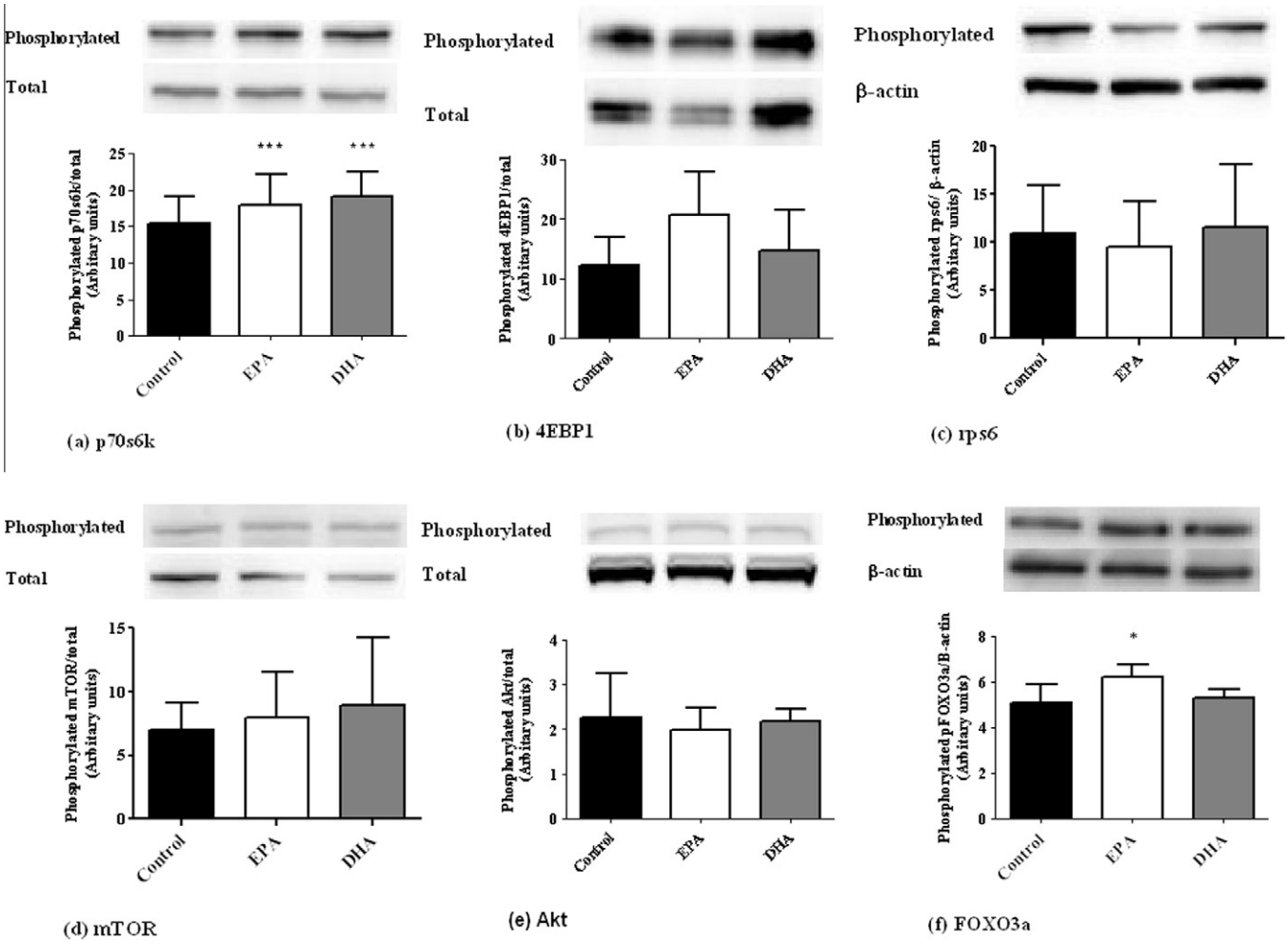

Fig. 3. Signalling pathways in control, EPA and DHA treatments in C2C12 myotubes. Data represent phosphorylated/total p70s6k$^{[Thr389]}$, 4EBP1$^{[Thr37/46]}$, rps6$^{[Ser235/236]}$, mTOR$^{[Ser2448]}$, Akt$^{[Ser473]}$ and FOXO3a$^{[Ser253]}$. Data are mean + SD ($n = 6$) and representative Western blots are shown. * Denotes a significant difference compared to the control treatment ($P < 0.05$, a one-way ANOVA with Bonferroni post hoc tests).

L-leucine stimulated protein synthesis. Furthermore we observed that individual EPA and DHA treatment augmented L-leucine stimulated p70s6k phosphorylation, independently of upstream regulators such as Akt and mTOR. The present findings also showed that incubation with individual EPA reduced protein breakdown, with no such effect of DHA.

In an early study, Palmer and Wahle (1987) reported that incubation with relatively low concentrations of EPA and DHA (0.2 and 1 μM) had no effect on the rate of isolated muscle protein synthesis in fasted rabbits [8]. In C2C12 cells it has been found that 50 μM EPA treatment caused a small stimulation in protein synthesis but had no protective effect after exposure to the catabolic stimuli PIF [9]. Recent investigations in humans have reported that EPA/DHA supplementation increase the rate of skeletal muscle protein synthesis during a hyperinsulinaemic–hyperaminoacidaemic clamp but not under fasted conditions [6,17]. Taken together these studies may suggest that the beneficial effects of EPA/DHA are only observed in response to anabolic stimuli such as feeding (i.e. amino acids and/or insulin). The distinct contributions of EPA and DHA in response to such an anabolic stimuli, however, remain unknown. In line with this the current study found that individual EPA treatment resulted in a ~25% increase in leucine stimulated protein synthesis compared with control condition, with no such effect with DHA treatment. These findings lead us to hypothesize that of the two fatty acids found in fish oil (EPA and DHA) that the stimulation of protein synthesis is likely to be due to EPA rather than DHA.

While changes in protein synthesis are likely to reflect protein accretion it is possible that concomitant changes in protein breakdown may nullify changes in protein synthesis. The importance of muscle protein breakdown in conditions such as sarcopenia is highlighted by the findings of an attenuated efficiency of insulin to prevent muscle protein breakdown in the elderly [4]. In study of Palmer and Wahle reported that in fasted rabbits neither EPA nor DHA (with lower concentrations) had any effect on the rate of protein breakdown in isolated forelimb muscles [8]. On the other hand in the present study we investigated the distinct effect of EPA and DHA on protein breakdown in C2C12 myotubes. We observed that incubation for 24 h with 50 μM EPA alone resulted in a ~22% reduction in protein breakdown compared to control condition, with no effect of DHA. Taken together with the stimulatory effect of EPA on protein synthesis (and data from other studies (e.g. [6]) it is possible that supplementation with EPA may be useful in the treatment of conditions where there is dysfunctional protein metabolism [18,19] or to enhance adaptations to stimuli such as resistance exercise, where alterations in protein metabolism are crucial [20].

To investigate the underlying mechanisms behind these effects we determined the distinct effects of EPA and DHA on signalling pathways in C2C12 myotubes. The current data demonstrates that

pre-incubation for 24 h with individual EPA and DHA (50 μM) enhanced L-leucine stimulated p70s6k phosphorylation (16% and 26% greater than that in control condition for EPA and DHA, respectively) in C2C12 myotubes. These findings agree with those of Smith et al. (2011) and our earlier findings in ageing rats, which further indicated that PI3K and PDK1 may be upstream mechanisms underlying the anabolic effects of fish oil [7]. Another interesting observation of the current study is that these observed increases in anabolic signalling do not readily reconcile with the observed changes in protein synthesis, similar findings to those previously reported [21]. This highlights the limitations in attempting to extend findings in anabolic signalling with physiological changes within the muscle cells themselves. When looking at markers the pathways of protein breakdown we measured FOXO3a and found that there was an increase in FOXO3a phosphorylation after incubation with EPA, but not DHA. This marker was chosen as the study of Rieu et al. found it to be crucial in the reductions in protein breakdown observed after treatment with ibuprofen in older rats [22]. Furthermore there are several research studies which have shown that the FOXO family of transcription factors regulate the two muscle specific ubiquitin E3 ligases, atrogin-1 and MuRF1 which can ubiquitinate proteins for degradation by the ubiquitin proteasome system. Both these proteins are upregulated in muscle wasting [23] and an increase in FOXO3a activation is sufficient to increase atrogin-1 and MuRF1 transcription [24,25]. Increasing the phosphorylation of FOXO3a at Ser253 causes exclusion from the nucleus to reduce transcription of atrogin-1 and MuRF1 genes which may be the mechanism underlying the reduction in protein breakdown as a result of EPA treatment. Further studies agree with this finding indeed it has been shown that the ubiquitin proteasome system, which can contribute to muscle protein breakdown [26,27], can be down regulated by EPA [10]. Moreover, Castillero et al. (2009) demonstrated that 12-days of EPA feeding (1 g/kg) decreased arthritis induced loss of body weight and muscle wasting, via diminished atrogin-1 and MuRF1 mRNA expression in arthritic rats [11]. What clearly remains to be established is how these alterations in signalling pathways and protein metabolism are brought about by EPA/DHA.

An early hypothesis was that fish oils would be beneficial for muscle protein metabolism would be due to their anti-inflammatory effects [28] and reducing the production of 2 series prostaglandins, known catabolic factors [29]. Indeed it is well established that both EPA and DHA have anti-inflammatory effects [30], but which has the most potent anti-inflammatory effect is not clear. It has been reported that EPA reduces TNF-α, IL-1β, IL-6, prostaglandin (PG)D2, and leukotriene (LT)B4 to a greater extent than DHA in human asthmatic alveolar macrophages [31]. Similarly, a more potent effect of EPA, compared to DHA, in decreasing the production of IL-2, IL-6, IL-10 and interferon-γ (IFN-γ) has been demonstrated in human lymphocytes [32–34]. On the other hand, Weldon et al. (2007) demonstrated that the effect of DHA was more pronounced, than that of EPA, in reducing TNF-α, IL-1β and IL-6 mRNA expression in human THP-1 macrophages [35]. Furthermore in a recent article Peng et al. (2012) demonstrated, in C2C12 cells, that while both EPA and DHA resulted in an inhibition of proliferation and cell growth, partly via a reduced phosphorylation of MAPK-ERK1/2 signalling pathways, DHA showed a greater inhibitory effect than EPA [36].

Recent work has however challenged the thesis that the improvements in protein metabolism associated with fish oil are due to their anti-inflammatory actions. Indeed in both animal and human studies anabolic effects have been found without concurrent reduction in systemic or circulating markers of inflammation [6,7]. Other potential mechanisms underlying the effects of EPA/DHA on muscle include enhanced insulin sensitivity which may increase the insulin-derived inhibition of muscle protein breakdown and also increase the delivery of amino acids to muscle via increases in blood flow [37], although it is not clear why these effects would have been observed in the current cell culture model. A further mechanism may relate to the increase in EPA/DHA incorporated into the skeletal muscle membranes altering the PI3K derived PIP3 potency in the activation of protein translation [7,38]. There is very little experimental evidence, at present, to support or refute these mechanisms and so further well mechanistic experiments are needed in this area.

Collectively, our findings in C2C12 myotubes demonstrated that EPA has a higher efficacy than DHA in augmenting L-leucine stimulated protein synthesis, anabolic signalling and to reduce protein breakdown. One could therefore suggest that fish oil supplementation containing a higher proportion of EPA than DHA could be the most efficacious in improving protein accretion in response to anabolic stimuli such as L-leucine/resistance exercise and could attenuate protein breakdown in ageing skeletal muscle. Further work in humans is clearly required to test this hypothesis.

Authors' contributions

SG conceived the study and revised the manuscript. TK carried out all data analyses and wrote the first draft of the manuscript. Both authors read and approved the final manuscript.

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