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ORIGINAL CONTRIBUTION

Fish oil positively regulates anabolic signalling alongside an increase in whole-body gluconeogenesis in ageing skeletal muscle

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Abstract
Purpose Fish oil, containing mainly long-chain n-3 polyunsaturated fatty acids (LCn-3PUFA), has been found to acutely stimulate protein synthesis and insulin-mediated glucose metabolism. However, the underlying mechanism and more prolonged effect of fish oil during ageing remain to be determined.
Methods Fish oil (EPAX6000; 49.6 % eicosapentaenoic acid, 50.4 % docosahexaenoic acid) or control oil (60 % olive, 40 % soy) supplementation was delivered, via chocolate-derived sweets, to rats for 8 weeks. Throughout the study, food intake and body weight were recorded and body composition was investigated using EchoMRI. During the last 40 min of a 6 h infusion, with labelled dextrose ([U-^{13}C]glucose) and amino acids ([1-^{13}C]phenylalanine), blood samples were collected to assess glucose and phenylalanine kinetics. Soleus and longissimus dorsi muscles were extracted for protein and mRNA analyses.
Results Fish oil had no effect on food intake or body composition. An increased whole-body glucose turnover, mainly accounted for via an increase in endogenous glucose production, was observed with fish oil feeding. No effects on whole-body phenylalanine turnover were observed. In longissimus dorsi, fish oil augmented the phosphorylation of phosphoinositide 3-kinase (PI3K)$^{[Tyr458]}$ ($P = 0.04$) and 70 kDa ribosomal protein S6 kinase (p70s6k)$^{[Thr389]}$ ($P = 0.04$). There were no differences in protein kinase B (Akt)$^{[Ser473]}$, mammalian target of rapamycin (mTOR)$^{[Ser2448]}$, protein phosphatase 2A (PP2A) 56 kDa regulatory B subunit γ (PP2A-B56-γ), forkhead box containing proteins O-subclass 3a (FOX03a)$^{[Ser253]}$ or inflammatory markers (Interleukin-6, Interleukin-1 β, tumour necrosis factor-α, and cyclooxygenase-2).
Conclusions Our data suggest that the fish oil may stimulate endogenous glucose production and increase anabolic signalling in ageing rats.

Keywords Ageing · Fish oil · p70s6k · Metabolism · Insulin sensitivity

Abbreviations

4EBP1	Eukaryotic initiation factor 4E (eIF4E) binding protein 1
Akt	Protein kinase B
COX2	Cyclooxygenase-2
DHA	Docosahexaenoic acid
EPA	Eicosapentaenoic acid
FoxO3a	Forkhead box containing proteins, O-subclass 3a
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GNG	Gluconeogenesis
IL-1β	Interleukin-1 beta
IL-6	Interleukin-6
LCn-3PUFA	Long-chain n-3 polyunsaturated fatty acids
LD	Longissimus dorsi
MCP1	Monocyte chemotactic protein 1
mTOR	Mammalian target of rapamycin

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p70s6k	70 kDa Ribosomal protein S6 kinase
PAI1	Plasminogen activator inhibitor 1
PDK1	Phosphoinositide-dependent protein kinase 1
PI3K	Phosphotidylinositol-3-kinase
PP2A-B56γ	Protein phosphatase 2A (PP2A) 56 kDa regulatory B subunit γ
Ptgs2	Prostaglandin-endoperoxide synthase 2
rps6	Ribosomal protein s6
TNF-α	Tumour necrosis factor alpha
TTR	Tracer to tracee ratio

Introduction

During ageing, there is a progressive decline in skeletal muscle mass (sarcopenia) beginning in middle age and affecting up to 50 % of those over 80 years of age [1]. This results in numerous deleterious effects including an impairment in daily tasks such as lifting, walking and stair climbing, an increased risk of falls [2] and metabolic disease [3]. In healthy humans, this reduction in muscle strength and capacity is associated with a decline in fibre numbers along with fibre atrophy, particularly in the type II glycolytic muscle fibres [4].

The underlying physiological mechanisms behind the age-associated decline in muscle mass are unclear. An imbalance in skeletal muscle protein turnover has been proposed as a potential cause [5]. Indeed, in humans, it has been shown that elderly muscle has an anabolic resistance to both nutrients [6] and exercise [7], with the effectiveness of insulin to limit muscle proteolysis also reduced [8]. Furthermore, as shown in an ageing rat model, ageing is associated with the development of chronic low-grade inflammation, which may in turn reduce protein synthesis and increase protein breakdown through alterations in anabolic/catabolic signalling pathways [9]. For example, in elderly men, the increases in phosphorylation of mTOR$^{[Ser2448]}$ and p70s6k$^{[Thr389]}$, key regulators of protein synthesis, are blunted in response to essential amino acids [6]. Furthermore, the phosphorylation of p70s6k, independently of mTOR, has been found to be reduced in response to amino acids and insulin in elderly human muscle [10].

Fish oil that contains large amounts of the two LCn-3PUFA EPA (20:5 n-3) and DHA (22:6 n-3) has previously been found to increase protein anabolism in young healthy steers [11] and to enhance insulin-induced glucose metabolism in the neonatal piglet [12]. Further reports have demonstrated that fish oil intake can conserve lean body mass in cancer patients [13] and suppress inflammation [14]. More recent work has found that an increase in LCn-3PUFA (4 g Lovaza/day containing 1.86 and 1.50 g/day of the ethyl esters of EPA and DHA, respectively) consumption results in an acute increase in protein synthesis during a single hyperaminoacidemic–hyperinsulinemic clamp in elderly humans, concurrent with an increased p70s6k phosphorylation [15]. Taken together, these findings indicate that fish oil, containing the LCn-3PUFA EPA and DHA, supplementation has the potential to reduce inflammation, improve protein synthesis and maintain lean body mass with age. The underlying mechanisms behind these effects and the more chronic effects of fish oil supplementation on muscle mass, in healthy ageing models, remain to be elucidated.

The aim of the present study therefore is to investigate the effects of fish oil supplementation on body composition and anabolic signalling pathways that are dysregulated in ageing skeletal muscle. Changes in energy substrate metabolism that may sustain any anabolic response were also monitored. In this study, we utilize 13-month-old rats, an age roughly equivalent to middle-aged humans where the onset of sarcopenia is noted [16].

Materials and methods

Animals and feeding

Thirty-two male Rowett strain Lister Hooded rats aged 13 months (mean weight 626.5 ± 30 g at baseline) were used in this study. Since the rats were bred over a long period of time, the Rowett strain was selected for its docility and facility to handle. Rats were divided in two groups (control and fish oil groups) with similar weight/body fat. All procedures were approved by the Animal Ethics Committee of the Rowett Research Institute under the auspices of the UK Animals (Scientific Procedures) Act 1986. Control- and fish oil-fed rats were both fed a basal semi-purified diet that met 100 % nutrient requirements and exceeded crude protein and metabolizable energy (ME) requirements by 10 % (pellet diet; Special Diet Services, Witham, Essex, UK). Lipids of the basal semi-purified diet were a mixture of olive and soy oils (40:60, weight basis) that provided the minimal nutritional requirements in n-3 [3 g/kg dry matter (DM)] and in n-6 fatty acids (23 g/kg DM) (NRC 1995); the n-6/n-3 ratio of the oil mixture was 7.6 (1–6 recommended).

The daily oil supplements, provided via chocolate-derived sweets containing the daily oils, consisted of a control oil ($n = 15$) composed of olive and soy oils (60:40, weight basis) or fish oil supplement ($n = 16$; EPAX 6000; menhaden oil consisting of 49.6 % EPA and 50.4 % DHA; EPAX, Oslo, Norway) fed daily over 8 weeks. The chocolate-derived sweets were a mixture of (g/100 g):

experimental oil (19.4; either control or EPAX oil), casein (5.4), icing sugar (41.5), cocoa (7.2), unsweetened baking chocolate (22, containing procyanidins and catechins and a relatively high antioxidant capacity) and vitamins and minerals (4.5, the same as used in the basal diet). The oil supplements were iso-energetic and represented a total daily portion of 2.5 g (8.7 kcal/kg of digestible energy). Nutrient content of the diet combined with the oil supplement represented 6.2 % protein, 10.5 % fat, 3.1 % ashes, 66 % carbohydrates and 16.3 MJ ME/kg, on DM basis. According to our previous studies, 6 weeks of fish oil supplementation was sufficient to achieve a 15 % threshold in muscle membrane phospholipids that induced insulin action [11, 12]. This period was extended to 8 weeks in the current study to ensure the achievement of as much EPA/DHA in the older muscle. Rats were housed singly in a controlled temperature room with a 12 h-light/12 h-dark cycle. Food and water were provided ad libitum. Intake was recorded daily with body weight recorded twice a week throughout the study.

Dextrose, amino acids and tracer infusion protocol

After a 16 h overnight fast, an insulin-stimulated state was generated via a continuous 360-min parenteral dextrose and amino acid infusion. On the morning of the measurements, the tail vein of the rats was cannulated under complete anaesthesia. Two blood backgrounds were sampled (2 × 300 µl). At time 0, a continuous i.v. dextrose infusion (77.27 µmol/kg min; Dextrose 20 %) labelled with [U-^{13}C]glucose (98 %; 8.95 µmol/kg min; CK GAS Products Ltd., Hampshire, UK) was performed over 6 h. Simultaneously, a complete amino acid solution that had a profile similar to rat muscle mixed proteins was continuously infused (17.58 µmol/kg min; [17]) labelled with [1-^{13}C]phenylalanine (99 %; 0.123 µmol/kg min; CK GAS Products Ltd., Hampshire, UK). Phenylalanine was selected as a tracer because it is not metabolized within skeletal muscle. A pilot study using similar rats showed that this dextrose infusion rate gave approximately a two-fold increase in blood glucose (5.1–9.2 mM) and insulin (66–140 pmol/l) concentrations and suppressed gluconeogenesis by 48 %. The amino acids used were pharmaceutical grade, and the solution was adjusted to pH 7.4. During the last 25 min of the infusion period, where steady-state measurements can be reliably obtained, six 500-µl samples were withdrawn. The total blood volume sample respected the UK guideline of use of laboratory animals. Immediately after parenteral feeding, rats were killed by i.v. injection of Pentobarbital Sodium (150 mg/kg). Soleus (SOL) and longissimus dorsi (LD) muscles were excised, immediately frozen in liquid N_2 and stored at −80 °C.

Measurements

Body composition

Body composition [i.e. total body fat (g)] was measured by the EchoMRI version 2004 (Echo Medical Systems, Houston, TX, USA.) every 4 weeks. Lean mass was calculated using total magnetic resonance imaging (MRI) fat mass (g) subtracted from body weight measured on scales (g) over 2 days previous to the MRI measure.

Plasma substrates and hormone

The concentration of glucose in fresh blood was immediately determined by glucose oxidase reaction (YSI 2300 STAT Plus). Blood samples were collected into tubes containing heparin, centrifuged (1,000×g at 4 °C for 10 min), and the plasma was stored at −80 °C for subsequent analyses. A discrete automated clinical analyzer (Kone Oyj, Espoo, Finland) was used for the analysis of triacylglycerols, low density lipoprotein (LDL) and high density lipoprotein (HDL) cholesterols using commercial kits (Labmedics; Salford, Manchester, United Kingdom). Insulin was measured in duplicate by using an enzyme-linked immunosorbent assay kit (Mercodia Uppsala, Sweden), with within-assay and between-assay CV of 5 and 3 %, respectively.

Plasma isotopic enrichments

Pentaacetate derivative of glucose was prepared as described by Fjeild et al. [18], and the isotopic enrichments of [^{13}C]glucose isotopomers was determined by gas chromatography–mass spectrometry (GC–MS) using a quadrupole instrument (Hewlett Packard HP5989A, Avondale, PA, USA). Methane chemical ionization was used in the positive ion mode, isotopic enrichments were measured by monitoring ions at m/z 331–337 to quantify the isotopic enrichment of $m+1$ to $m+6$ glucose isotopomers according to the method of Haymond and Sunehag [19]. Plasma phenylalanine was separated on a cation exchange resin column (AGW50 resin, Bio-Rad) and converted to their n-propyl ester heptafluorobutyramide and analysed by GC–MS using a Hewlett Packard HP5989A Engine (Hewlett Packard, Avondale, PA, USA) as described previously [20]. The respective m/z monitored was m/z 383 and 384.

Western blotting

Muscle was homogenized 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). After centrifugation, the supernatant was removed and total protein concentration was measured by Bicinchoninic acid (BCA) assay. Samples were then diluted with 3× Laemmlli sodium dodecyl sulphate (SDS) sample buffer and homogenization buffer to give a final working concentration of 2 μg/μl. Forty to eighty μg protein of each sample was then loaded and separated by 12 % sodium dodecyl sulphate–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). 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-PI3K$^{[Tyr199,Tyr458]}$, phospho-phosphoinositide-dependent protein kinase 1(PDK1)$^{[Ser241]}$, phospho-FOX03a$^{[Ser253]}$, β-actin from Cell Signalling Technology (Danvers, MA, USA.) and PP2A-B56-γ (H-40) from Santa Cruz Biotechnology (Delaware, CA, 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 signalling bands were quantified by using ImageJ 1.42q (NIH, USA).

Ribonucleic acid (RNA) extraction and complementary deoxyribonucleic acid (cDNA) synthesis

Muscle tissue was homogenized on ice in cold TRIzol reagent (Invitrogen/Life Technologies, Carlsbad, CA, USA). The RNA concentration was measured by NanoDrop ND-1000 spectrophotometer software version 3.3 (NanoDrop Technologies Inc, Wilmington, DE, USA.) at a wavelength of 260/280 nm and cDNA synthesized using the SuperScript™ II RT (Invitrogen/Life Technologies, Carlsbad, CA, USA).

Quantitative real-time PCR (qPCR)

The messenger RNA (mRNA) expression of markers of inflammation was measured by quantitative real-time PCR. Quantitative real-time PCR was performed using TaqMan® probes. The rat mRNA sequences for Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), β-actin, 18S ribosomal RNA (18s rRNA), TNF-α, IL-6, IL-1β, and COX2 PCR primers for TaqMan® probes were designed from the Universal ProbeLibrary, ProbeFinder version 2.45 for rat (Universal ProbeLibrary, Roche). Primer sequences are as follows: GAPDH forward 5′-AGCTGGTCATCAATGGGAAA-3′, reverse; 5′-ATTTGATGTTAGCGGGATCG-3′; β-actin forward 5′-CCCGCGAGTACAACCTTCT-3′, reverse 5′-CGTCATCCATGGCGAACT-3′, 18S ribosomal RNA (18s rRNA) forward 5′-AATCAGTTATGGTTCCTTTGTCG-3′, reverse 5′-GCTCTAGAATTACCACAGTTATCCAA-3′, TNF-α forward 5′-TGAACTTCGGGGTGATCG-3′, reverse 5′-GGGCTTGTCACTCGAGTTTT-3′; IL-6 forward 5′-CCCTTCAGGAACAGCTATGAA-3′, reverse 5′-ACAACATCAGTCCCAAGAAGG-3′; IL-1β forward 5′-TGTGATGAAAGACGGCACAC-3′, reverse 5′-CTTCTTCTTTGGGTATTGTTTGG-3′; Ptgs2 or COX2 forward 5′-CTACACCAGGGCCCTTCC-3′, reverse 5′-TCCAGAACTTCTTTTGAATCAGG-3′. Primers were purchased from Sigma-Aldrich (Gillingham, Dorset, UK). The reactions were carried out in 20 μl reaction mix containing of 10 μl 2× LightCycler 480 Probes MasterMix (FastStart Taq DNA polymerase, reaction buffer, dNTP mix with dUTP instead of dTTP, 6.4 mM $MgCl_2$), 6.4 μl RNase-free water, 3 μl cDNA, 0.2 μl specific UPL probes and 0.2 μl 20 μM of each primer. For each sample, the reactions were carried out in triplicate. Briefly, pre-incubation was set for 10 min at 95 °C followed by 45 PCR cycles consisting of a 10-s denaturing step at 95 °C, and a 30-s amplification step at 60 °C. qPCR was analysed by using LightCycler® 480 system version 1.5 (Roche Diagnostics, Mannheim, Germany). The amount of each target mRNA was determined using the relative amount to GAPDH, β-actin and 18S rRNA expression.

Kinetics calculations

Calculation of glucose kinetics

Total plasma glucose appearance rate was calculated from the plasma enrichment of [U-$^{13}C_6$]glucose, and gluconeogenesis was estimated using Mass Isotopomer Distribution Analysis (MIDA) of plasma glucose according to the reciprocal pool model of Haymond and Sunehag. Corrections for the isotopic contribution of natural abundance and distribution of the isotopomers in the tracer were conducted according to the matrix approach [21]. Glucose enrichments are reported as mol per cent excess.

Calculations

Kinetics were assessed during steady-state conditions over the last 25 min of the 6 h i.v. nutrition of glucose and amino acids.

$$\text{Exogenous glucose entry rate} = \text{dextrose infusion rate};$$

$$\text{Total plasma glucose appearance rate} = [E_i/E_p \times \text{IR}]$$

where E_i is the $^{13}C_6$-enrichment of the infusate; E_p is the $^{13}C_6$-enrichment in plasma, and IR is the infusion rate of [U-^{13}C]glucose (μmol/kg min). Under steady-state conditions, glucose appearance rate = glucose disappearance (utilization) rate.

Whole-body flux of glucose =
Total plasma glucose appearance rate − Tracer IR
Glucose endogenous production rate =
Total plasma glucose appearance rate − GIR

where GIR is the glucose infusion rate, which is the sum of labelled and unlabelled glucose infused, that is, [U-$^{13}C_6$]glucose + unlabelled dextrose.

The fractional gluconeogenesis, that is, gluconeogenesis as a percentage of total plasma glucose appearance rate, was calculated according to the reciprocal pool model by use of [U-^{13}C]glucose (Haymond and Sunehag [19]). This model is based on the assumption that [U-^{13}C]glucose infused is the only precursor of endogenously synthesized labelled glucose. However, loss and exchange of labelled carbons across the TCA cycle simultaneous to entry of unlabelled and labelled glucose species into the glucose molecule endogenously synthesized occur, and the reciprocal pool model accounts for this phenomenon. This model only requires the analyses of glucose isotopomers to calculate the fractional gluconeogenesis.

$$\text{Fractional GNG} = \frac{\sum_1^5 M_i}{\sum_1^6 M_i} \times \frac{\sum_1^5 {}^{12}CM_i}{\sum_1^5 {}^{13}CM_i}$$

where M_i are the isotopomers of glucose representing the fraction of all of the labelled species of glucose derived from gluconeogenesis, that is, glucose molecules labelled with ^{13}C on 1 through 6 of their carbons. The ratio of entry rates of the ^{12}C and ^{13}C carbons in the glucose species labelled as a result of the process of gluconeogenesis is represented by $^{13}C_{M_i}$ and $^{12}C_{M_i}$, that is, glucose molecules originating from gluconeogenesis labelled on 1 through 5 of their carbons. Haymond and Sunehag [19] provides the detailed model.

Gluconeogenesis rate = Fractional gluconeogenesis
× Glucose endogenous production rate
Glycogenolysis = Glucose endogenous production rate
− Gluconeogenesis rate

Calculation of phenylalanine kinetics Phenylalanine isotopic enrichments are reported as molar tracer to tracee ratio (TTR).

Exogenous entry rate = Parenteral phenylalanine infused
Whole−body flowrate = $[E_i/E_p \times IR] - IR$

where E_i is the $^{13}C_1$ enrichment of the phenylalanine; E_p is the isotopic enrichment of phenylalanine in plasma, and IR is the infusion rate of [1-^{13}C]phenylalanine (μmol/kg min).

Endogenous flow (protein breakdown) rate
= Whole−body flowrate − Exogenous entry rate

Statistical analyses

Data are expressed as means and standard deviation (SD) and were analysed by using SPSS 18.0 software. A two-way ANOVA (time and supplement group) was performed to test the effect of oil supplementations on food intake, body weight, lean mass and fat mass at every time point of recording. Differences in the response of signalling proteins, in the level of mRNA expression of key inflammatory markers, plasma substrates and metabolism parameters between groups were carried out by independent *t* tests. The level of statistically significant difference was set as $P < 0.05$. The control group data were obtained from 15 rats because of one rat death prior to the study completion.

Results

Effects of fish oil on food intake, body weight and body composition

Over the 2-month study period, a two-way ANOVA revealed that food intake (measured daily) and body weight (measured twice a week) decreased. The analysis also found that total lean mass decreased and total fat mass increased (both measured every 4 weeks) over the study period. There were no significant differences in food intake, body weight and body composition at any time point between groups (Table 1). However, when the change in total lean mass, over the study period, was calculated there was a trend for a smaller decrease in the fish oil group (-18.8 ± 5.1 g vs. -9.7 ± 3.0 g, $P = 0.10$).

Effects of fish oil on blood parameters

There were no differences between the groups in plasma glucose (Table 2). In the fasting state, plasma LDL, HDL and triacylglycerols were lower in the fish oil compared with the control group. In the Dextrose/Insulin Infused state, LDL and HDL were lower in the fish oil compared with the control group, with no difference in triacylglycerols.

Table 1 Effect of fish oil on food intake, body weight and body composition

	Dietary treatment		P^a		
	Control	Fish oil	Time	Group	Time × group
Food intake (g/day)			0.001	0.38	0.76
At initial	26.63 (3.11)	25.04 (3.49)			
At 1 month	18.46 (1.72)	17.99 (2.46)			
At 2 months	14.55 (1.92)	14.97 (2.21)			
Body weight (g)			0.001	0.33	0.85
At initial	633.80 (28.31)	619.75 (32.21)			
At 1 month	654.06 (32.62)	642.28 (34.67)			
At 2 months	654.95 (36.44)	646.55 (36.68)			
Total fat mass (g)			0.001	0.83	0.41
At initial	93.61 (17.95)	97.29 (18.78)			
At 1 month	122.07 (14.70)	129.03 (23.05)			
At 2 months	131.56 (14.37)	132.42 (21.50)			
Total lean mass(g)			0.001	0.25	0.41
At initial	540.64 (32.18)	522.46 (32.38)			
At 1 month	527.00 (32.16)	513.24 (29.18)			
At 2 months	521.43 (35.39)	511.22 (28.72)			

$n = 15$ for control- and $n = 16$ for fish oil-fed rats. Data are means (±SD)

[a] P values were determined by a two-way ANOVA to test the effect of oil supplementations on food consumption and animal mass and composition at every time point of recording

Table 2 Effect of fish oil on blood parameters

	Dietary treatment		
	Control	Fish oil	P
Plasma glucose (mmol/l)			
Fasting state[a]	11.7 (1.30)	11.4 (1.08)	0.59
Dextrose/insulin infused state[b]	13.0 (3.34)	11.7 (1.75)	0.25
Plasma lipids (mmol/l)[c]			
LDL cholesterol			
Fasting state	0.27 (0.09)	0.15 (0.05)	0.0007
Dextrose/insulin infused state	0.25 (0.07)	0.13 (0.04)	<0.0001
HDL cholesterol			
Fasting state	1.76 (0.23)	1.38 (0.18)	0.0003
Dextrose/insulin infused state	1.52 (0.20)	1.13 (0.21)	<0.0001
Triacylglycerols			
Fasting state	2.37 (0.48)	1.85 (0.60)	0.03
Dextrose/insulin infused state	1.84 (0.54)	1.73 (0.45)	0.55

Estimate of insulin concentration: 112 μU/ml in control- and 140 μU/ml in fish oil-fed rats (SD 31; $n = 10$ fasting state and $n = 9$ Dextrose/Insulin Infused state) obtained from incomplete data set, see "Materials and methods" for details

Data are the mean (±SD)

[a] Fasting state values: $n = 13$ for control- and $n = 11$ for fish oil-fed rats

[b] Dextrose/Insulin Infused state values: $n = 12$ for control- and $n = 12$ for fish oil-fed rats

[c] Fasting state values: $n = 13$ for control- and $n = 11$ for fish oil-fed rats. Dextrose/Insulin Infused state values: $n = 13$ for control- and $n = 14$ for fish oil-fed rats

Effects of fish oil on insulin-stimulated whole-body glucose and phenylalanine kinetics

There was no difference between the two groups in glucose mass isotopomer enrichments (Table 3). Although glucose isotopic enrichments were not altered with fish oil supplementation, glucose whole-body flux normalized for body weight was increased. This occurred alongside a greater endogenous glucose production, mainly accounted for by a higher gluconeogenesis rate compared to the control group. There were no differences between groups in exogenous glucose entry rate, fractional gluconeogenesis or glycogenolysis (Table 3). Neither the isotopic enrichment nor the whole-body flux of phenylalanine normalized for body weight was altered by dietary treatments (Table 4). Exogenous entry rate and plasma concentration of phenylalanine were similar between groups, as a result phenylalanine endogenous flux from protein breakdown was unaltered with fish oil supplementation.

Effects of fish oil on anabolic signalling responses

Fish oil supplementation had no effect on the phosphorylation of $Akt^{[Ser473]}$, $mTOR^{[Ser2448]}$, $4EBP1^{[Thr37/46]}$ and $p70s6k^{[Thr389]}$ in the soleus muscle (data not shown). As there was no difference in phosphorylation within the Akt-mTOR-p70s6k pathways in the soleus muscle, further protein and mRNA analysis was not carried out in these samples.

By contrast, in LD (predominantly type II fibres), fish oil produced a 44.6 ± 16.4 % increase ($P = 0.02$) in the phosphorylation of $p70s6k^{[Thr389]}$ compared to the control group (Fig. 1). Furthermore, there was a trend ($P = 0.08$) for a 20.41 ± 11.12 %, higher phosphorylation of

Table 3 Effect of fish oil on whole-body glucose kinetics

	Dietary treatment		*P*
	Control	Fish oil	
Glucose mass isotopomer enrichments (MPE %)			
$m + 0$	86.6 (0.7)	86.6 (0.6)	0.98
$m + 1$	1.46 (0.2)	1.64 (0.3)	0.12
$m + 2$	1.08 (0.2)	1.21 (0.3)	0.15
$m + 3$	1.20 (0.3)	1.29 (0.3)	0.40
$m + 4$	0.25 (0.03)	0.25 (0.04)	0.95
$m + 5$	0.64 (0.03)	0.61 (0.05)	0.15
$m + 6$	8.77 (0.6)	8.40 (0.8)	0.21
Glucose kinetics (μmol/kg·min)			
Whole-body flux	90.3 (4.22)	97.3 (9.71)	0.02
Exogenous glucose entry rate	80.3 (1.20)	81.5 (2.15)	0.13
Endogenous production	18.7 (4.19)	24.8 (9.11)	0.04
Fractional gluconeogenesis	0.49 (0.08)	0.56 (0.12)	0.12
Gluconeogenesis Rate	9.6 (3.27)	14.9 (7.92)	0.03
Glycogenolysis	9.07 (1.05)	9.9 (1.62)	0.12

$n = 13$ for control- and $n = 14$ for fish oil-fed rats. Data are the mean (±SD)

Table 4 Effect of fish oil on whole-body phenylalanine kinetics

	Dietary treatment		*P*
	Control	Fish oil	
Phenylalanine concentration (μmol/ml)	97.7 (25.90)	92.6 (16.59)	0.56
Isotopic enrichment TTR (mol tracer/100 mol tracee)			
$m + 1$	4.7 (1.55)	4.6 (1.88)	0.83
Phenylalanine kinetics (μmol/kg·min)			
Whole-body flux	2.51 (0.50)	2.47 (0.38)	0.81
Exogenous entry rate	1.02 (0.27)	0.99 (0.29)	0.86
Endogenous flux	1.50 (0.51)	1.48 (0.35)	0.90

$n = 13$ for control- and $n = 14$ for fish oil-fed rats. Data are the mean (±SD)

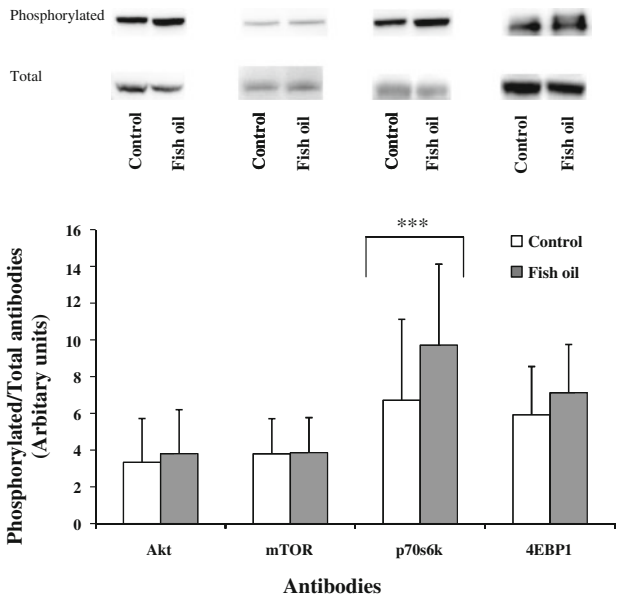

Fig. 1 Anabolic signalling pathways in longissimus dorsi muscle in control and fish oil groups after dextrose/amino acids infusion. Data represent phosphorylated/total Akt[Ser473], mTOR[Ser2448], p70s6k[Thr389] and 4EBP1[Thr37/46]. Data are mean + SD (control: $n = 15$; fish oil: $n = 16$) and representative western blots are shown. ***Significant difference compared with the control group ($P < 0.05$)

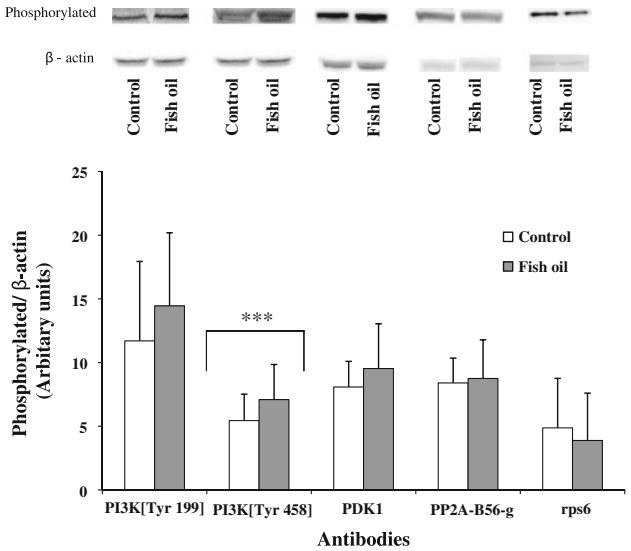

Fig. 2 Upstream and downstream signalling pathways of p70s6k in longissimus dorsi muscle in control and fish oil groups after dextrose/amino acids infusion. Data represent phosphorylated PI3K[Tyr199], PI3K[Tyr458], PDK1[Ser241], rps6[Ser235/236] and PP2A-B56-γ (H-40)/β-actin. Data are mean + SD (control: $n = 15$; fish oil: $n = 15$) and representative western blots are shown. ***Significant difference compared with the control group ($P < 0.05$)

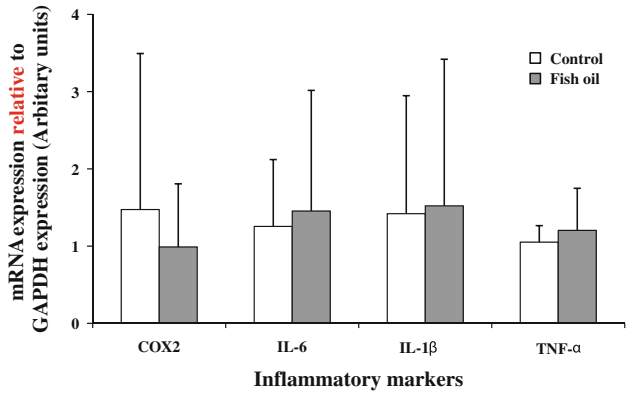

Fig. 3 mRNA expression of key inflammatory markers in longissimus dorsi muscle in control and fish oil groups. Data represent mRNA expression level of COX2, IL-6, IL-1β and TNF-α/GAPDH expression. Data are mean + SD (control: $n = 8$; fish oil: $n = 8$)

4EBP1[Thr37/46] in the fish oil compared to the control group. There was no difference between groups in the phosphorylation of mTOR[Ser2448] and its upstream effector Akt[Ser473] in LD. The fish oil-enriched diet resulted in a 30.25 ± 13.1 % greater ($P = 0.04$) phosphorylation of PI3K[Tyr458] compared to the control group (Fig. 2). There was also a trend for a greater phosphorylation of PI3K[Tyr199] (23.5 ± 12.66 %, $P = 0.11$) and PDK1[Ser241] (17.95 ± 13.10 %, $P = 0.09$) compared to the control group. There was no difference in the phosphorylation of PP2A-B56-γ, rps6[S235/236] or FOX03a[Ser253] between groups.

Effects of fish oil on mRNA expression of key inflammatory markers

After the 8 week supplementation period, there was no difference in mRNA expression of COX2, IL-6, IL-1β and TNF-α in the LD muscle between the two groups (Fig. 3). For simplicity, we have shown only inflammatory marker expression relative to GAPDH, with the results the same when expressed relative to β-actin and 18S rRNA.

Discussion

The current study has demonstrated that 8 weeks fish oil supplementation increased whole-body glucose flux, which was mainly accounted by an increased endogenous glucose production. Furthermore fish oil supplementation increased the phosphorylation of PI3K and p70s6k in LD muscles, independently of any changes in mTOR or Akt signalling. There were no significant effects of fish oil supplementation on food intake or body composition.

Body composition and glucose kinetics

Previous studies have reported that fish oil supplementation, high in EPA and DHA, can improve weight maintenance in cancer patients [13]. The current data have shown

that fish oil supplementation has no effect on food intake or body weight/composition in ageing rats, although a trend for a greater preservation of lean mass was observed. It is pertinent to note that the measurement of lean mass by MRI, as in the current study, also measures skeletal, blood, visceral organs and water mass. It will, therefore, be important to investigate the specific effect of fish oil supplementation on changes in muscle mass specifically. In support of an anabolic role in muscle, recent work by Smith et al. [15] demonstrated that fish oil supplementation stimulates protein synthesis during a hyperaminoacidemic–hyperinsulinemic clamp in elderly participants. A single measure of protein synthesis, however, does not provide the whole picture with regard to protein accretion, as there may also be concurrent alterations in protein breakdown. To investigate this, the current study measured whole-body phenylalanine kinetics, with no differences between groups, indicating that protein breakdown was not altered with the consumption of fish oil. This observation is further supported by our other findings that FOXO3a phosphorylation, which has a major involvement in proteolysis [22], was not altered by fish oil supplementation.

Any changes to muscle anabolism would be an energy-dependent process and in the fed state glucose is the primary substrate that sustains metabolic pathways. We, therefore, investigated the effects of fish oil on glucose kinetics and found that glucose whole-body flux rate was increased by 7.8 % with fish oil supplementation. This is a relatively small effect, especially considering the inter-animal variation observed, and the physiological significance of this increase remains to be established. Whilst we did not measure muscle glucose uptake in the current study, several researchers have demonstrated that skeletal muscle glucose uptake is increased after feeding with fish oil [23] and that fish oil can protect against saturated fatty acid-induced insulin resistance [24]. On the other hand, Gillam et al. [25] reported that n-3 fatty acids diet had no positive effect on insulin resistance in the young fa/fa Zucker rat model and indeed the literature on the effects of fish oil on glucose metabolism and insulin action is inconsistent (for review see [26]).

In the current study, since exogenous entry and blood glucose levels were not different between groups, endogenous glucose production was enhanced, in response to an increase in peripheral demand for glucose. The glucose kinetics model employed allows one to further delineate that glycogenolysis was not responsible for sustaining the greater endogenous production but that gluconeogenesis was increased to compensate for the supplemental glucose demand in the rats fed fish oil. Ageing is known to reduce insulin sensitivity of glucose metabolism in lean rats/humans, and both whole-body disposal rate and endogenous production are behind this dysregulation [27]. The current study indicates that it is likely that fish oil feeding is effective in improving muscle insulin sensitivity through the improvements in glucose whole-body metabolic flow. Further work looking specifically at the effect of fish oil on skeletal muscle glucose and protein metabolism will be crucial to confirm and identify the magnitude of this effect.

Signalling pathways

It has previously been suggested that a decrease in inflammation would relieve the inhibition of intracellular signalling pathways, such as the mTOR pathway, and thus increase protein accretion. With regard to intracellular signalling, we found that the fish oil-enriched diet had no effect on the activation of key proteins in anabolic signalling pathways in soleus muscle, mainly composed of type I muscle fibres. However, in LD, predominantly type II muscle fibres, 8-week fish oil supplementation amplified a hyperglycemic-hyperinsulinemic–euaminoacidemic clamp-induced activation of p70s6k, which is a key regulator in the initiation step of protein synthesis [28]. This occurred without any changes in the upstream proteins such as Akt and mTOR and the catabolic factor FOXO3a. Previous studies have also demonstrated that fish oil enhances the phosphorylation of anabolic signalling pathways in skeletal muscle. Indeed we have previously demonstrated that in neonatal piglets and in young steers, fish oil supplementation stimulates protein metabolism [11, 12] alongside an increased activation of the Akt-mTOR-p70s6k pathway. Furthermore, the study of Smith et al. demonstrated that the increase in protein synthesis after fish oil supplementation occurred alongside an increase in the activation of the p70s6k pathway independently of Akt activation [15, 29]. These findings agree with those of the current study that fish oil supplementation increases p70s6k phosphorylation, which is known to be dysregulated in ageing skeletal muscle, independently of changes in mTOR [10]. The mechanisms behind the fish oil supplementation-related activation of p70s6k have, however, not been investigated in any previous studies. The family of signal transduction enzymes PI3K catalyses the conversion of PIP2 to PIP3 in the inner leaflet of the plasma membrane [30], and increasing the unsaturation of the membrane has been hypothesized to increase the activation of PDK1 [31]. Once activated PDK1 can phosphorylate p70s6k independently of mTOR [32]. The current data have shown that fish oil supplementation increases the phosphorylation of $PI3K^{[l\text{-}Tyr458]}$. Similarly, Taouis et al. [33] reported that n-3PUFA supplementation, containing a mixture of both short-chain and long-chain n-3PUFA, maintained PI3K activity in young male rat muscle. This indicates that increasing the n-3 content in plasma membranes can increase PI3K activity.

What remains to be elucidated is the physiological relevance of these, relatively small, fish oil-mediated changes in cell signalling. Indeed it is not clear whether the 45 % greater p70s6k phosphorylation, after fish oil supplementation, is sufficient to stimulate protein synthesis and if so what is the extent of this stimulation. For example, after 6 weeks exercise training, in rats, a ~14 % hypertrophy was associated with a ~350 % increase in p70s6k phosphorylation [34]. It is possible therefore that the modest anabolic effect of fish oil on p70s6k is not of a great enough magnitude to result in detectable changes in lean mass over the relatively short 2-month study period employed in the current study. Further long-term studies are, therefore, warranted.

The LCn-3PUFA EPA and DHA, found in fish oil, can be incorporated into membrane phospholipids [35], at the expense of the n-6PUFA arachidonic acid. This leads to a reduction in the arachidonic acid-derived inflammatory markers, such as PGE-2, which are thought to be more potent than n-3-derived mediators such as PGE-3 [36]. In the current study, however, no difference in markers of inflammation within skeletal muscle was observed between the groups of rats, suggesting a reduction in inflammation may not be the mechanism through which fish oil increase anabolic signalling pathways in our rat model. This is supported by recent work by Smith et al. (2011) who found no alterations in circulating inflammatory markers with fish oil supplementation in elderly humans, although protein synthesis was increased. With respect to the animal model employed in the current study, evidence of chronic low-grade inflammation in 13- to 15-month-old rats is lacking. Some studies have demonstrated low-grade inflammation and body weight loss/impaired protein metabolism in rats aged over 20 months old [9, 37]. However, even though we observed a loss in lean mass in 13-month-old rats, it remains crucial to determine the effect of fish oil supplementation during acute or chronic inflammatory conditions, and whilst there are some promising data in cancer models [13, 38], several other conditions with an inflammatory component, such as sarcopenia, may benefit from increased fish oil consumption.

Limitations

Regarding the current study there are limitations. Firstly, we studied rats aged between 13 and 15 months; however, in Rieu et al. (2009) rats of 25 months old were studied. These older rats had high concentrations of the inflammatory markers IL-6, IL-1β, monocyte chemotactic protein 1 (MCP1) and plasminogen activator inhibitor 1 (PAI1) and more progressive muscle loss and would provide information on the effect of fish oil in sarcopenia. Secondly, relative to the life span of a rat, our 2-month supplementation period was rather short. Therefore, to determine whether fish oil supplementation is helpful in protection from age-related sarcopenia, more long-term (i.e. 10–25 months in rats) studies are required. Thirdly, due to the lack of muscle-specific measurements, we cannot confirm that the trend for total lean mass conservation in fish oil group represents skeletal muscle mass preservation, although the responses in signalling would suggest this. Further work investigating the effects of fish oil supplementation in ageing is needed, looking at myofibrillar protein synthesis and breakdown.

Conclusions

In conclusion, 8 weeks fish oil supplementation increases whole-body glucose flux and anabolic signalling pathways in rats. Whole-body glucose flux was augmented via an increase in endogenous glucose production and gluconeogenesis. Consistently with previous results, fish oil supplementation increased protein anabolic signalling pathways, specifically in type II muscle fibres, without any regulatory effect exerted via inflammation process.

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Conflict of interest The authors have no conflicts of interest.

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