J Physiol 592.6 (2014) pp 1341–1352

The Journal of Physiology

Omega-3 supplementation alters mitochondrial membrane composition and respiration kinetics in human skeletal muscle

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Key points

- Following fish oil supplementation, omega-3 fatty acids are incorporated into cellular membranes, which may affect lipid–protein interactions and therefore the function of embedded proteins.
- As the components of the electron transport chain required for oxidative phosphorylation are contained in the mitochondrial membrane, omega-3 supplementation may alter metabolic function.
- We supplemented male participants for 12 weeks with fish oil [eicosapentaenoic acid (EPA) and docosahexanoic acid (DHA)] and analysed mitochondrial function and reactive oxygen species (ROS) emissions in permeabilized muscle fibres from the vastus lateralis muscle.
- Supplementation incorporated EPA and DHA into mitochondrial membranes, but did not result in changes in maximal mitochondrial respiratory function or pyruvate respiration kinetics.
- However, the apparent K_m for ADP was decreased following supplementation, and was independent of creatine, changes in the protein content of ADP synthase or ANT transporters.
- The propensity for ROS emissions increased with omega-3 supplementation, although there were no changes in markers of lipid or protein oxidative damage.
- These results demonstrate that omega-3 supplementation improves mitochondrial ADP kinetics, suggesting post-translational modification of existing proteins.

Abstract Studies have shown increased incorporation of omega-3 fatty acids into whole skeletal muscle following supplementation, although little has been done to investigate the potential impact on the fatty acid composition of mitochondrial membranes and the functional consequences on mitochondrial bioenergetics. Therefore, we supplemented young healthy male subjects ($n = 18$) with fish oils [2 g eicosapentaenoic acid (EPA) and 1 g docosahexanoic acid (DHA) per day] for 12 weeks and skeletal muscle biopsies were taken prior to (Pre) and following (Post) supplementation for the analysis of mitochondrial membrane phospholipid composition and various assessments of mitochondrial bioenergetics. Total EPA and DHA content in mitochondrial membranes increased ($P < 0.05$) ~450 and ~320%, respectively, and displaced some omega-6 species in several phospholipid populations. Mitochondrial respiration, determined in permeabilized muscle fibres, demonstrated no change in maximal

DOI: 10.1113/jphysiol.2013.267336

substrate-supported respiration, or in the sensitivity (apparent K_m) and maximal capacity for pyruvate-supported respiration. In contrast, mitochondrial responses during ADP titrations demonstrated an enhanced ADP sensitivity (decreased apparent K_m) that was independent of the creatine kinase shuttle. As the content of ANT1, ANT2, and subunits of the electron transport chain were unaltered by supplementation, these data suggest that prolonged omega-3 intake improves ADP kinetics in human skeletal muscle mitochondria through alterations in membrane structure and/or post-translational modification of ATP synthase and ANT isoforms. Omega-3 supplementation also increased the capacity for mitochondrial reactive oxygen species emission without altering the content of oxidative products, suggesting the absence of oxidative damage. The current data strongly emphasize a role for omega-3s in reorganizing the composition of mitochondrial membranes while promoting improvements in ADP sensitivity.

(Received 24 October 2013; accepted after revision 3 January 2014; first published online 6 January 2014)
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Abbreviations AA, arachidonic acid; ALA, α-linoleic acid; ANT, adenine nucleotide translocase; CL, cardiolipin; DHA, docosahexanoic acid; EPA, eicosapentaenoic acid; IMF, intermyofibrillar; LA, linoleic acid; miCK, mitochondrial creatine kinase; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PUFA, polyunsaturated fatty acid; ROS, reactive oxygen species; SM, sphingomielin; SS, subsarcolemmal

Introduction

Skeletal muscle is a key tissue influencing metabolic homeostasis, as it represents ∼40% of body weight, is highly adaptable and has a variable metabolic rate. Within muscle, the transport of both carbohydrate and fatty acids across membrane bilayers represents a key regulatory step influencing fuel selection and overall metabolism. Omega-3 supplementation, particularly with fish oils enriched with eicosapentaenoic acid (EPA; 20:5n-3) and DHA (docosahexanoic acid; 22:6n-3), results in significant incorporation of polyunsaturated fatty acids (PUFAs) into numerous membrane phospholipid species within whole skeletal muscle (Yamaoka *et al.* 1988; Andersson *et al.* 2002; Owen *et al.* 2004; Dangardt *et al.* 2012).

Alterations in dietary fatty acid composition are therefore thought to alter the thickness, stiffness and fluidity of the lipid bilayer, impacting metabolism. To date, studies examining the effect of EPA and DHA supplementation on skeletal muscle membrane composition have been limited to whole muscle measurements. Nonetheless, DHA represents a primary fatty acid in skeletal muscle mitochondrial phospholipids (Fiehn *et al.* 1971; Tsalouhidou *et al.* 2006) and it has been suggested that DHA is required for the organization and function of membrane proteins (Infante & Huszagh, 2000). This may be particularly important in mitochondria, where electron transfer is tightly coupled between the complexes of the embedded electron transport chain. Therefore, accumulation of omega-3 PUFAs within mitochondrial membranes may affect mitochondrial bioenergetics, a suggestion supported by the recent observation that the unsaturation index of mitochondrial membranes in rodents is positively associated with rates of palmitate oxidation (Holloway *et al.* 2012). However, it remains to be determined if mitochondrial membrane composition is altered in human skeletal muscle following omega-3 PUFA supplementation.

Direct evidence suggesting that mitochondrial oxidative phosphorylation is improved following omega-3 PUFA supplementation does not exist in human skeletal muscle. However, it has been shown that rats fed omega-3 PUFAs utilized less oxygen for a given twitch force both during and recovering from multiple bouts of prolonged contraction (Peoples & McLennan, 2010). This indirectly suggests improved mitochondrial efficiency with omega-3 intake, resulting either from greater content of the electron transport complexes, or from enhanced kinetics of existing proteins. Although mitochondrial changes have not been investigated in detail in skeletal muscle following omega-3 PUFA supplementation, initial work in rat heart mitochondria demonstrated increased *in vitro* ATP synthase activity consistent with an improvement in mitochondrial respiratory function following supplementation. However, direct assessments of maximal mitochondrial respiration suggested that omega-3 supplementation decreases (Yamaoka *et al.* 1988) or has no effect (Khairallah *et al.* 2012; Lanza *et al.* 2013) on mitochondrial respiration in either heart or skeletal muscle, complicating interpretations. One limitation of these studies is that mitochondrial function has been exclusively determined in the presence of saturating ADP concentrations. As such, these studies reflect the *in vitro* capacity for oxidative phosphorylation and probably not the *in vivo* situation, as ADP transport appears to be

highly regulated (Perry *et al.* 2012). From a physiological standpoint, it may be more appropriate to examine mitochondrial function in the presence of submaximal concentrations of ADP, although this has yet to be considered following omega-3 PUFA supplementation. The metabolic consequence of altering omega-3 PUFA availability therefore remains ambiguous.

The primary purpose of the current study was to investigate the effects of prolonged fish oil supplementation on mitochondrial content, mitochondrial bioenergetics and submaximal respiratory substrate kinetics in human vastus lateralis muscle. Specifically, we aimed to determine if omega-3 PUFA supplementation altered (1) mitochondrial membrane composition, (2) maximal ADP and pyruvate-supported respiration, (3) the dynamic response and/or sensitivity of the electron transport chain to ADP and pyruvate, and (4) the propensity for mitochondrial reactive oxygen species (ROS) production. We found that omega-3 PUFA supplementation resulted in pronounced omega-3 incorporation into mitochondrial membranes without altering maximal ADP-supported respiration. In contrast, mitochondrial ADP sensitivity, submaximal ADP-stimulated respiration and maximal mitochondrial ROS emission were all increased following fish oil supplementation. The improved sensitivity of mitochondria to ADP was independent of creatine kinase involvement, changes in the protein content of ATP synthase or ADP transporters. The current data therefore provide direct evidence that omega-3 PUFA supplementation improves mitochondrial respiratory function in human skeletal muscle.

Methods

Subjects and supplementation

Eighteen healthy, recreationally active men (22.7 ± 0.8 years, 82.1 ± 2.2 kg and 182.8 ± 1.5 cm) were recruited in two rounds for this study. Mitochondria were isolated for phospholipid analysis from the first nine participants, while ADP respiratory kinetics in the presence of creatine were determined in the last nine participants. All other assessments of mitochondrial bioenergetics were determined in all 18 participants. The two groups of participants ($n = 9$ per group) completed the study separated by 12 months. Importantly, mitochondrial ROS, ADP respiratory kinetics and pyruvate respiratory kinetics, were measured in both groups, and showed the same statistical responses. Therefore, we have reported data for these measurements on all individuals ($n = 18$).

All participants were free of disease and were not on prescription medication or supplements. Subjects were placed on Omega-3 Complete (Jamieson Laboratories Ltd, Windsor, Canada) on a dose of five pills per day (omega-3 intake: 2 g EPA, 1 g DHA per day) for a 12 week period. Prior to (Pre) and following (Post) the intervention two biopsies were taken from the vastus lateralis. A small portion of the first biopsy was immediately used for the preparation of permeabilized fibres (described below) with the remaining portion flash-frozen for Western blotting. The second biopsy was used for the isolation of mitochondria. This study was approved by the Research Ethics Boards of the University of Guelph (Guelph, Ontario) and McMaster University (Hamilton, Ontario), and conforms to the Declaration of Helsinki.

Mitochondrial isolation

Mitochondrial subsarcolemmal (SS) and intermyofibrillar (IMF) populations were isolated by differential centrifugation as described previously (Campbell *et al.* 2004) with minor modifications. Muscle was quickly minced and diluted in 1 ml mg^{-1} isolation buffer [100 mM sucrose, 100 mM KCl, 50 mM Tris-HCl, 1 mM KH_2PO_4, 0.1 mM EGTA, 0.2% bovine serum albumin (BSA), 1 mM ATP; pH 7.4] and homogenized using a polytron with a teflon head. SS mitochondria underwent centrifugation at 800 *g* for 10 min and the supernatant was pelleted at 10,000 *g* for 10 min before being washed in MiR05, (detailed below) and spun again at 10,000 *g* for 10 min. After the initial 800 *g* spin, the IMF mitochondrial pellet was resuspended 10-fold in isolation buffer and treated with 0.25 μg protease/mg muscle for 5 min before being diluted 10-fold in isolation buffer and spun again at 5000 *g* for 5 min. The IMF pellet was resuspended in isolation buffer and pelleted at 10,000 *g* for 10 min before being washed in MiR05, and spun again for 10 min at 10,000 *g*. The final pellets for both SS and IMF isolations were combined and resuspended in 100 μl S&M solution (225 mM mannitol, 75 mM sucrose, 10 mM Tris-HCl, 0.1 mM EDTA; pH 7.4). Mitochondria were further purified using a percoll gradient and pooled for analysis.

Mitochondrial lipid analysis

The membrane phospholipid composition of isolated mitochondria was determined using gas chromatography compared to an internal 15:0 standard as previously described (Bonen *et al.* 2004). Briefly, the phospholipid fraction was separated by thin-layer chromatography. The gel bands corresponding to the standards were scrapped off the plates, transferred into fresh tubes and then transmethylated in 14% methanolic boron trifluoride (Sigma) at 100°C for 30 min. Individual fatty acid methyl esters were identified and quantified according to the retention times of standards by gas liquid chromatography

[Hewlett-Packard 5890 Series II gas chromatograph, HP Varian CP-SIL capillary column (50 m × 0.25 mm internal diameter) and flame-ionization detector (Agilent Technologies, CA, USA]. Total phospholipid content was estimated as the sum of the particular fatty acid species of the assessed fraction and expressed in nanomoles per gram of tissue.

Preparation of permeabilized fibres

A small portion of each biopsy was placed in ice-cold BIOPS (50 mM MES, 7.23 mM K_2EGTA, 2.77 mM CaK_2EGTA, 20 mM imidazole, 0.5 mM dithiothreitol, 20 mM taurine, 5.77 mM ATP, 15 mM PCr and 6.56 mM $MgCl_2.H_2O$; pH 7.1) and separated under a microscope into bundles using fine-tipped forceps as described previously (Perry *et al.* 2012). Fibre bundles were then treated with 30 μg ml^{-1} saponin for 30 min at 4°C, then either washed for 15 min in MiR05 respiration buffer (0.5 mM EGTA, 10 mM HH_2PO_4, 110 mM sucrose and 1 mg ml^{-1} fatty acid-free BSA; pH 7.1) for respiration analysis, or buffer Z (105 mM K-MES, 30 mM KCl, 1 mM EGTA, 10 mM KH_2PO_4, 5 mM $MgCl_2.H_2O$, 0.005 mM pyruvate, 0.002 mM malate with 5 mg ml^{-1} BSA; pH 7.4) for measurements of H_2O_2 emission as described below.

Mitochondrial respiration

Measurements of O_2 consumption were performed in MiR05 respiration medium on prepared permeabilized fibres using an Oxygraph-2K (Oroboros Instruments, Innsbruck, Austria) at 37°C in the presence of 25 μM blebbistatin as previously described (Perry *et al.* 2011). Mitochondrial kinetics were analysed using three separate titration protocols. ADP kinetics were analysed in the presence and absence of 20 mM creatine, separately. Both ADP titrations were initiated with 5 mM malate and 10 mM pyruvate, and ADP was titrated at various concentrations. Pyruvate kinetics were determined in the presence of 5 mM ADP, 5 mM malate and 20 mM creatine, while pyruvate was titrated at various concentrations. Then, 10 mM glutamate and 10 mM succinate were added following the pyruvate protocol to determine maximum mitochondrial respiration. All fibres were recovered from the respirometer, freeze-dried and weighed for normalization to muscle bundle weight. The apparent K_m for pyruvate and ADP was determined as previously described (Perry *et al.* 2012).

H_2O_2 emission

Mitochondrial H_2O_2 emission was measured in buffer Z fluorometrically (Lumina, Thermo Scientific, Waltham, MA, USA) at 37°C in the presence of 25 μM blebbistatin. Cuvettes contained 10 μg ml^{-1} oligomycin, 10 μM Amplex Red (Invitrogen, Carlsbad, CA, USA), 0.5 U ml^{-1} horseradisch peroxidase and 40 U ml^{-1} superoxide dismutase. The reaction was initiated by the addition of 10 mM succinate and H_2O_2 emission was calculated from the slope (absorbance min^{-1}) and made relative to fibre dry weight as performed previously (Anderson *et al.* 2009).

Western blotting

Whole muscle was homogenized in lysis buffer, diluted to 1 μg μl^{-1}, and loaded equally for α-tubulin (Abcam, Cambridge, MA, USA), ANT1 (MitoSciences, Eugene, OR, USA), ANT2 (Abcam), 4HNE (Alpha Diagnostics, San Antonio, TX, USA) and OXPHOS (MitoSciences), and for use of the Oxyblot protein oxidation detection kit (Millipore, Billerica, MA, USA). Proteins were separated by SDS-PAGE, transferred to polyvinylidene difluoride membranes, and incubated in blocking solution, primary antibody, and the corresponding secondary antibody as specified by the supplier. Membrane proteins were detected by enhanced chemiluminesence (ChemiGenius2 Bioimaging system, SynGene, Cambridge, UK).

To confirm homogeneity in the OXPHOS content between fibre bundles, Western blotting was performed on recovered fibres as previously described (Murphy, 2011) and modified (Lally *et al.* 2013). Briefly, permeabilized fibres were digested in 5 μl μg^{-1} digestion buffer [10% glycerol (w/v), 5% β-mercapoethanol (v/v), 2.3% SDS (w/v) in 62.5 mM Tris-HCl, pH 6.8] for 15 min at 65°C with gentle shaking. For determination of OXPHOS protein content, 10 μl was loaded onto a 12% SDS-polyacrylamide gel and run and detected as described above. Due to the sensitive nature of complex IV, the heating preparation made this protein undetectable with this method.

Statistics

All values are presented as mean ± SEM. The apparent K_m values for pyruvate and ADP were determined using Prism (GraphPad Software, Inc., La Jolla, CA, USA), as published previously (Smith *et al.* 2013). All pre- and post-supplementation measures were analysed for significance using a paired *t* test with the α-value set to $P < 0.05$.

Results

Mitochondrial phospholipid composition

The purity of our mitochondrial isolations was first confirmed by the presence of mitochondrial proteins as well as the absence of sarcoplasmic reticulum (SERCA) and plasma membrane (caveolin-1) proteins

Table 1. Absolute quantification of phospholipid species in isolated mitochondria before and after fish oil supplementation

		18:3 (ALA)	20:5 (EPA)	22:6 (DHA)	18:2 (LA)	20:4 (ALA)	Total phospholipids
PC	Pre	0.98 ± 0.07	1.06 ± 0.07	0.70 ± 0.14	98.43 ± 8.18	12.68 ± 1.36	242.20 ± 15.78
	Post	0.82 ± 0.06	9.35 ± 4.51	4.51 ± 0.46*	83.95 ± 4.69*	16.78 ± 1.29*	243.27 ± 8.30
PE	Pre	0.44 ± 0.07	2.72 ± 0.45	5.49 ± 1.14	19.43 ± 3.94	63.65 ± 7.45	188.40 ± 18.54
	Post	0.36 ± 0.12	10.61 ± 2.52*	15.85 ± 2.27*	17.62 ± 2.30	51.15 ± 5.05*	178.69 ± 15.96
CL	Pre	0.68 ± 0.13	0.57 ± 0.05	0.11 ± 0.02	81.10 ± 13.53	0.73 ± 0.10	101.53 ± 13.84
	Post	0.56 ± 0.06	0.50 ± 0.03	0.12 ± 0.03	63.30 ± 9.36	0.54 ± 0.04*	81.71 ± 6.18
SM	Pre	0.18 ± 0.04	ND	0.066 ± 0.023	1.71 ± 0.37	0.27 ± 0.04	21.51 ± 1.99
	Post	0.15 ± 0.04	ND	0.029 ± 0.010	1.43 ± 0.41	0.22 ± 0.03	12.30 ± 1.05*
PI	Pre	0.20 ± 0.02	0.33 ± 0.08	0.15 ± 0.05	2.55 ± 0.17	9.62 ± 1.02	49.91 ± 3.75
	Post	0.21 ± 0.01	0.36 ± 0.06	0.19 ± 0.04	2.38 ± 0.24	8.79 ± 0.76	48.95 ± 3.29
PS	Pre	0.24 ± 0.04	ND	0.45 ± 0.06	2.78 ± 0.44	0.75 ± 0.13	33.15 ± 6.39
	Post	0.22 ± 0.04	ND	1.32 ± 0.23*	2.07 ± 0.21	0.63 ± 0.11	34.41 ± 4.57

Mitochondrial membrane phospholipid species before (Pre) and after (Post) 12 weeks of fish oil supplementation. Alpha linoleic acid (ALA), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), linoleic acid (LA) and arachidonic acid are reported in various phospholipid species, including: phosphatidylcholine (PC), phosphatidylethanolamine (PE), cardiolipin (CL), sphingomielin (SM), phosphatidylinositol (PI) and phosphotidylserine (PS). Values represent means ± SEM, and are expressed as nmol mg^{-1} protein. $n = 9$. *Significantly ($P < 0.05$) different from pre-supplementation.

(Fig. 1). The effect of n-3 fish oil supplementation on mitochondrial membrane phospholipid composition was thus determined in highly purified mitochondrial samples. Specifically, we examined the total abundance of α-linolenic acid (ALA, 18:3n-3), EPA (20:5n-3) and DHA (22:6n-3), as well as their incorporation into phosphatidylcholine (PC), phosphatidylethanolamine (PE), cardiolipin (CL), sphingomielin (SM), phosphatidylinositol (PI) and phosphatidylserine (PS). Although the omega-3 intervention did not alter total mitochondrial membrane phospholipid content (Table 1), the EPA and DHA present in the fish oil supplements increased total membrane incorporation of these PUFAs ~4.5- and ~3-fold, respectively, without altering the content of ALA (Fig. 2*A*). EPA increased ~8.8- and ~3.9-fold in PC and PE fractions, respectively, while either remaining unchanged or undetectable in other phospholipid fractions (Fig. 2*A*). DHA incorporation increased ~6.4- and ~2.9-fold in PC and PE fractions, respectively, while also increasing ~2.9-fold in PS.

We also analysed mitochondrial membranes for the presence of linoleic acid (LA, 18:2n-6) and arachidonic acid (AA, 20:4n-6) phospholipid species. Total LA content was reduced to 83% of Pre and decreased to ~85% in the PC fraction (Fig. 2*B*). While total AA content was not altered, AA accumulation in PE decreased to ~80% of Pre, and, by contrast, increased ~1.3-fold in the PC fraction.

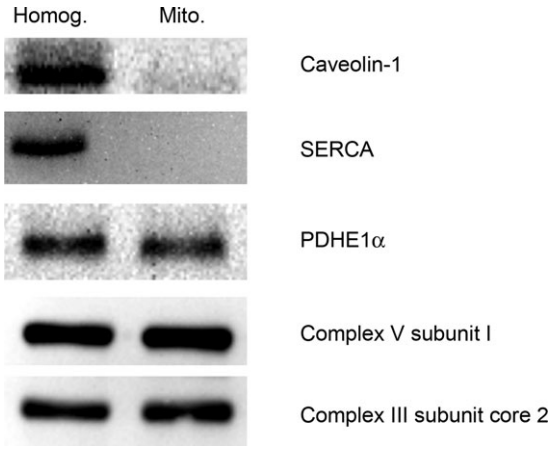

Figure 1. Purity of mitochondrial isolation
Representative Western blots of whole muscle lysates (Homog.) and isolated mitochondria (Mito.) showing the presence of known mitochondrial proteins and the absence of plasma membrane (caveolin-1) and sarcoplasmic reticulum (SERCA) proteins. Mitochondrial proteins included the E1α subunit pyruvate dehydrogenase (PDHE1α) and subunits of complex 3 and 4 of the electron transport chain. These were loaded as 30 μg of whole muscle and 5 μg of mitochondria.

Mitochondrial respiration and kinetics

Given that omega-3 supplementation resulted in incorporation of omega-3 PUFAs into the mitochondrial membrane, we aimed to determine if this impacted mitochondrial respiratory function. In coupled mitochondria (respiratory control ratio ~6), we first analysed maximal pyruvate-stimulated respiration, as well as maximal complex I (pyruvate+glutamate)- and complex I+II (pyruvate+glutamate+succinate)-stimulated respiration, and found no differences following omega-3 supplementation (Fig. 3*A*). These data suggest that omega-3 fish oil supplementation does not alter the

capacity of the electron transport chain. This observation was further confirmed by Western blotting for subunits of the electron transport chain in whole muscle (Fig. 3*B*), which showed no difference in OXPHOS protein content between time-points.

Given the absence of changes in maximal substrate-supported respiration, we next examined the dynamic response of mitochondria to small changes in pyruvate availability. Similar to maximal substrate-supported respiration, omega-3 supplementation did not alter the kinetics of pyruvate, as the apparent sensitivity (K_m) (Fig. 3*D*) and maximal respiration (Fig. 3*C*) induced by pyruvate were not altered.

We next determined if omega-3 supplementation altered ADP kinetics, by performing ADP titrations both in the presence and in the absence of creatine. While titrating ADP produced the same maximal respiration as observed in the pyruvate titrations before supplementation, fish oil consumption increased maximal respiration in the presence of creatine (Fig. 4*A*), but not in the absence of creatine (Fig. 4*C*). Given the unexpected finding of increased maximal respiration, we digested the actual fibre bundles used to determine ADP sensitivity for Western blotting of OXPHOS subunits to ensure mitochondrial content was not higher. Similar to the whole muscle (Fig. 2*D*), various markers

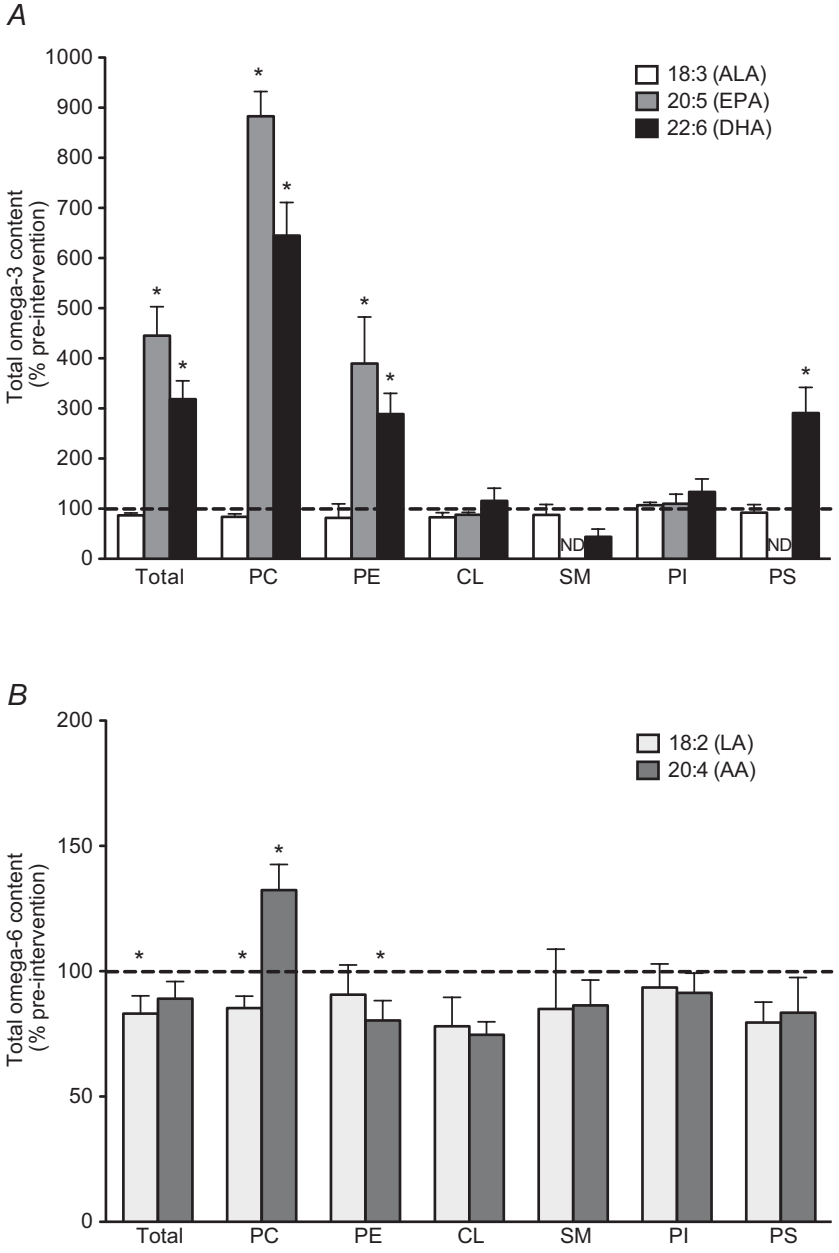

Figure 2. Fish oil supplementation and PUFA content in isolated mitochondrial membranes of human skeletal muscle
Fish oil supplementation results in changes in omega-3 (*A*) and omega-6 (*B*) PUFA content in isolated mitochondrial membranes of human skeletal muscle. The content of α-linolenic acid (ALA), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), linoleic acid (LA) and arachidonic acid (AA) are reported in various phospholipid species, including: phosphatidylcholine (PC), phosphatidylethanolamine (PE), cardiolipin (CL), sphingomielin (SM), phosphatidylinositol (PI) and phosphatidylserine (PS). Values represent means ± SEM, and are expressed as a percentage of pre-supplementation. $n = 9$. *Significantly ($P < 0.05$) different from pre-supplementation. ND; Not detected.

of OXPHOS were not increased in fibre bundles used for ADP titrations (Fig. 4*E*). In addition to changes in maximal respiration, ADP titrations revealed an increased sensitivity to ADP, as shown by significant decreases of 34 and 20% in the apparent K_m following supplementation in both the presence (Fig. 4*A* and *B*) and absence (Fig. 4*C* and *D*) of creatine, respectively. These data suggest that the increased ADP sensitivity following supplementation is probably mitochondrial creatine kinase (miCK) independent, potentially involving ATP synthase or adenine nucleotide translocase (ANT). However, the content of ATP synthase was not altered at the whole muscle level or in fibre bundles (Figs 3*B* and 4*E*). Furthermore, we also determined the protein content of ANT1 and ANT2 (Fig. 4*F*) and found no differences following supplementation. Therefore, these data suggest that the improvement in ADP sensitivity is probably a result of post-translational modification of these proteins.

H_2O_2 emission and oxidative stress

Omega-3 incorporation into the mitochondria probably alters membrane fluidity and the ease by which electron slippage and superoxide production occurs. Given the observation of increased respiration at submaximal ADP concentrations, we speculated that this could occur as a result of increased coupling efficiency, which may augment the propensity for mitochondria to produce ROS. We therefore determined mitochondrial ROS emission following fish oil supplementation and showed

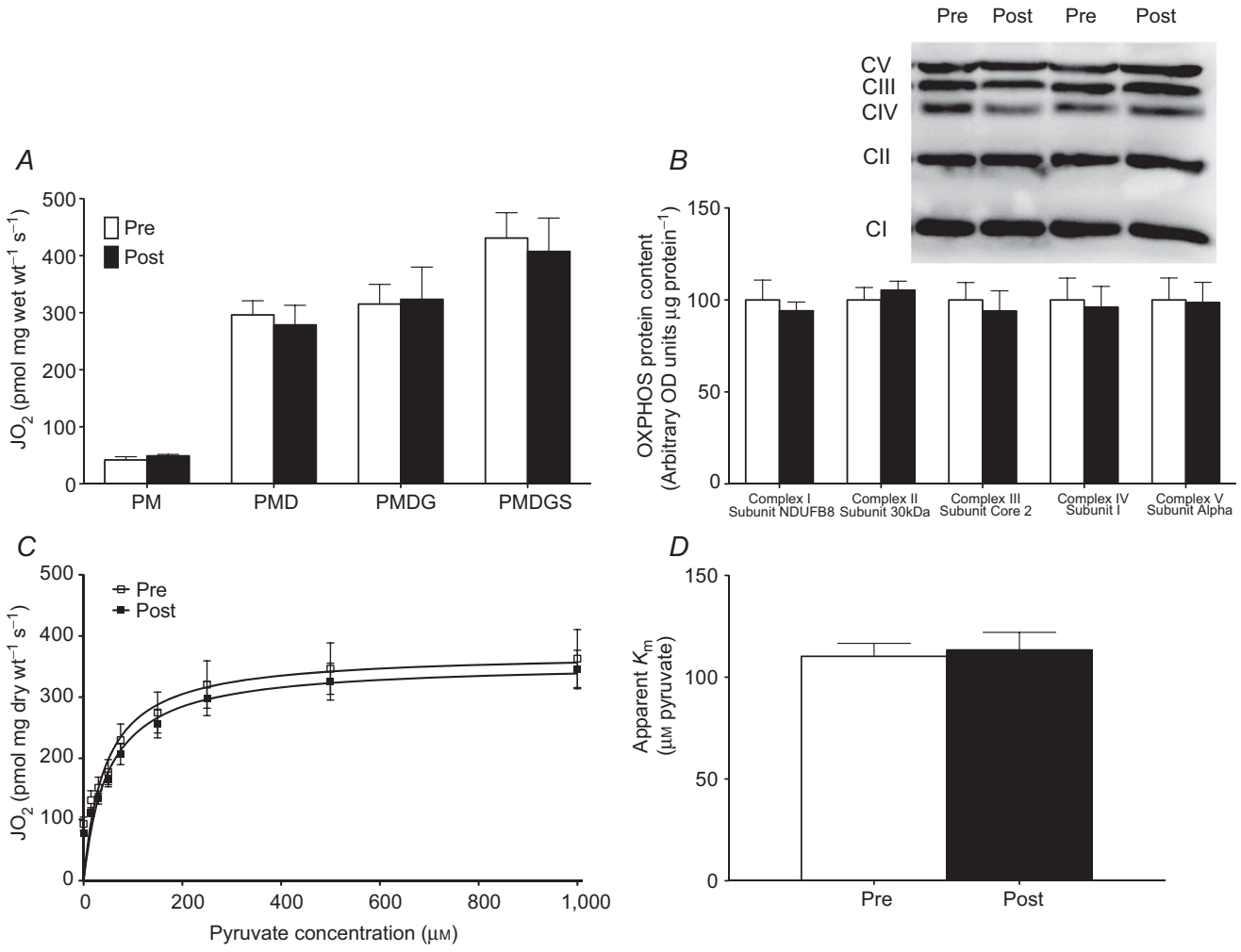

Figure 3. Results of omega-3 incorporation into mitochondrial membranes
Omega-3 incorporation into mitochondrial membranes does not alter maximal mitochondrial respiration (*A*), the expression of oxidative phosphorylation (OXPHOS) proteins (*B*), mitochondrial pyruvate kinetics, (*C*) or the apparent K_m for pyruvate (*D*). Values were compared before (Pre) and after (Post) 12 weeks of omega-3 supplementation. Non-ADP-stimulated (pyruvate + malate; PM), ADP-stimulated (PMD), maximal complex I supported respiration (PMDG) and maximal coupled respiration (PMDGS) were not different after supplementation. Values represent means ± SEM. $n = 18$.

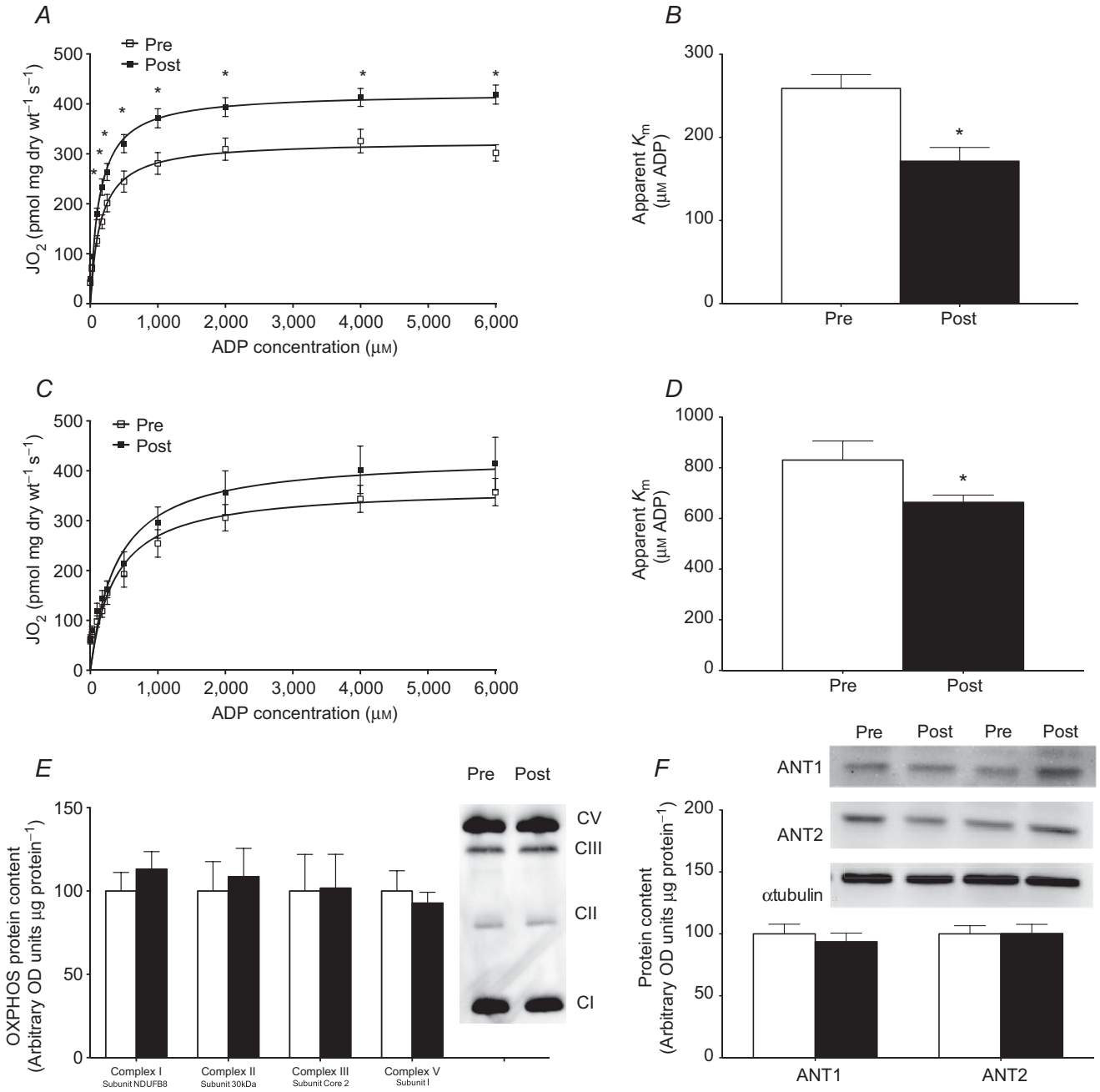

Figure 4. Omega-3 incorporation into mitochondrial membranes in the presence and absence of creatine
Omega-3 incorporation into mitochondrial membranes altered ADP respiratory kinetics in the presence (*A*, *B*) and absence (*C*, *D*) of creatine. Michaelis–Menten kinetic curves were generated in the presence (*A*) and absence (*C*) of creatine. The ADP concentration required to elicit half-maximal respiration (apparent K_m) was determined in the presence (*B*) and absence (*D*) of creatine. Values were compared before (Pre) and after (Post) 12 weeks of omega-3 supplementation. Fibre bundles were recovered from the respiration chamber and used to determine the expression of oxidative phosphorylation (OXPHOS) proteins (*E*). The protein content of adenine nucleotide translocase (ANT1, ANT2) was determined by Western blotting (*F*). Values represent means ± SEM. n = 9–18. *Significantly ($P < 0.05$) different from Pre.

that the propensity for succinate-induced mitochondrial H_2O_2 emission was increased ~1.3-fold following supplementation (Fig. 5*A*). As this supports a larger capacity for ROS generation with omega-3 consumption, we also examined products of oxidative stress. The presence of lipid peroxidation as determined by 4HNE immunoblotting (Fig. 5*B*) and the abundance of protein carbonyl groups (Fig. 5*C*) were not different after supplementation in muscle homogenates. Therefore, the enhanced capacity for H_2O_2 formation does not result in increased oxidative damage.

Discussion

The present study investigated the impact of fish oil supplementation on mitochondrial phospholipid composition, bioenergetics and respiratory function in human skeletal muscle. Our novel findings demonstrate that omega-3s are robustly incorporated into mitochondrial membranes following supplementation, but without altering maximal mitochondrial function. By contrast, mitochondrial ADP sensitivity, submaximal ADP-stimulated respiration, and maximal mitochondrial ROS emission were all increased following fish oil supplementation.

It is well established that altering fatty acid composition of the diet can effect membrane composition in both rodent heart (Charnock *et al.* 1986) and skeletal muscle (Owen *et al.* 2004), and utilizing various dosages and time frames, omega-3 PUFA supplementation has also been shown to result in omega-3 accumulation in whole muscle membranes from human tissue (Andersson *et al.* 2002; Stark *et al.* 2002; Dangardt *et al.* 2012; Smith *et al.* 2013). Here we show that fish oil supplementation results in robust incorporation of omega-3 PUFAs into human muscle mitochondrial membranes and displaces omega-6 PUFAs from some phospholipid species, supporting previous work conducted in rat heart (Khairallah *et al.* 2012) and liver (Pehowich, 1999). Specifically, in mitochondria, we observed EPA and DHA content increases of 3- to 9-fold in PC and PE fractions, which coincided with a decrease in LA abundance. The increased incorporation of omega-3 PUFAs into the mitochondrial membranes is remarkably similar to previous reports on whole muscle samples (Stark *et al.* 2002). However, in contrast to previous work in rodents suggesting that DHA is efficiently incorporated into CL (Khairallah *et al.* 2012), omega-3 supplementation did not alter the composition of CL in this study. At the plasma membrane, increased AA has been hypothesized to contribute to inflammatory eicosanoids. Therefore, while it is possible that a similar relationship exists at the mitochondria, currently how mitochondria are impacted by changes in omega-6 content in individual phospholipid species is unknown. In addition, the present data represent the phospholipid species contained within both the inner and the outer mitochondrial membrane, and therefore it remains unknown what changes specifically occur in the more biologically relevant inner mitochondrial membrane. CL is a mitochondrial phospholipid composed

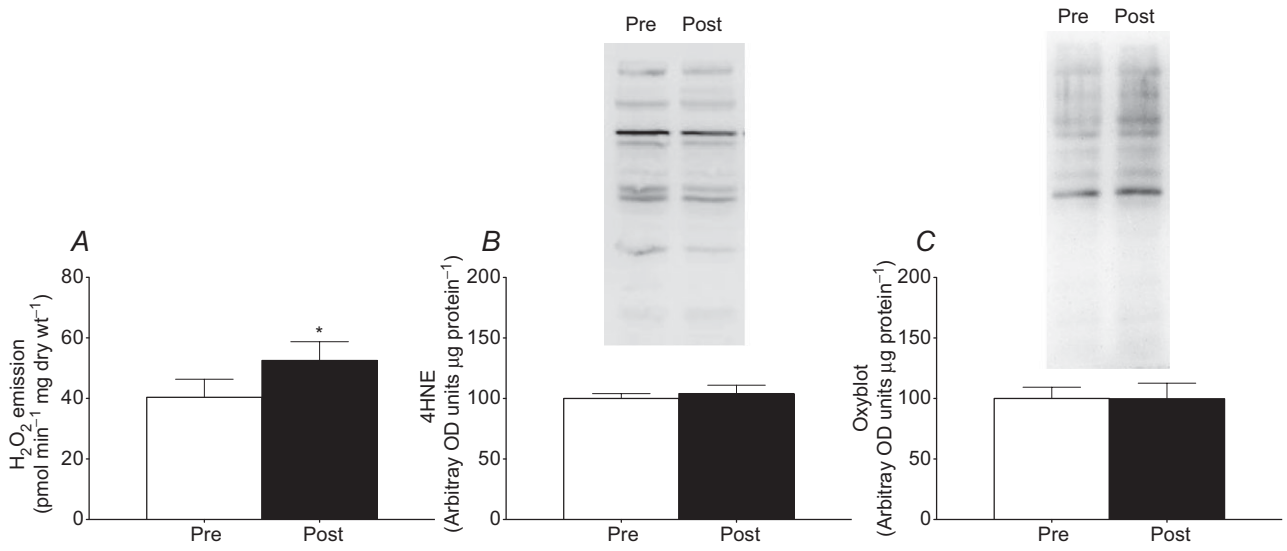

Figure 5. ROS after omega-3 incorporation into mitochondrial membranes
Omega-3 incorporation into mitochondrial membranes increased maximal succinate-induced reactive oxygen species (ROS) emission (*A*) without altering markers of oxidative stress. Specifically, 4-hydroxynonenal (4HNE; *B*) and protein carbonylation (Oxyblot; *C*) were not different before (Pre) or after (Post) 12 weeks of omega-3 supplementation. Values represent means ± SEM. $n = 18$. *Significantly ($P < 0.05$) different from Pre.

primarily of LA (Minkler & Hoppel, 2010). CL is unique to the inner membrane and is vital to the integrity of the membrane proteins, as it aids in the formation of contact sites on the inner and outer membranes and is closely associated with the proteins of the electron transport chain (Sparagna & Lesnefsky, 2009). Although we observed no changes in omega-3 incorporation into CL, there were strong trends for reductions in both LA ($P = 0.058$) and AA ($P = 0.10$) content in CL following supplementation. The consequence of this is unknown, although previous work supports an important role for CL in the failing heart, as LA supplementation aids in attenuating losses in CL content and improving mitochondrial dysfunction in cardiomyopathies (Sparagna *et al.* 2007; Mulligan *et al.* 2012). Therefore, small reductions in CL omega-6 PUFA content may have biological relevance, although the metabolic consequences of alterations in CL content presently remain ambiguous.

In the current study we provide evidence that maximal ADP-stimulated respiration in permeabilized muscle fibres was not altered by omega-3 intake, corroborating previous work in rodents (Lanza *et al.* 2013). We also examined the mitochondrial response to small changes in substrate availability. Following the titration of pyruvate, no change was detected in either maximal respiration (V_{max}) or the apparent affinity (K_m) under conditions of saturating ADP. This suggests that following omega-3 supplementation, electron acceptance through complex I, flow to complex IV, and ultimately electron acceptance by oxygen were not altered. Where complex I substrates are seldom limiting physiologically, rapid changes in free ADP concentrations probably dictate fluctuations in mitochondrial respiration *in vivo*. While ATP and ADP are thought to freely diffuse across concentration gradients through ANT, the majority of the energy transfer from the matrix to the cytoplasm occurs through miCK-dependent phosphate shuttling in oxidative muscle (Aliev *et al.* 2011; Guzun *et al.* 2012). We unexpectedly found that titrating ADP in the presence of creatine resulted in a robust increase in V_{max}, which was independent of changes in OXPHOS protein expression. In addition, although not significantly different, the same basic trend is visually depicted in the absence of creatine. No explanation currently exists for this observation, but it probably does not reflect changes in ADP transport directly, given that ADP concentrations are saturating. Interestingly, in both the presence and the absence of creatine, the apparent K_m for ADP decreased following omega-3 supplementation. This occurred in the absence of altered ANT1, ANT2, or ATP synthase protein content. These data suggest that increased omega-3 PUFA incorporation into mitochondrial membranes directly alters ADP sensitivity, or perhaps indirectly affects post-translational modifications of ANT isoforms or ATP synthase. In combination with the unaltered pyruvate kinetics, these data suggest that the mitochondria respond differently following fish oil supplementation to situations of low membrane potential (i.e. ADP added before pyruvate titration) and high membrane potential (i.e. pyruvate added before ADP titrations). The apparent fish oil-induced improvement in ADP sensitivity may be speculated to improve the efficiency with which ATP resynthesis occurs during exercise. This may explain previous reports demonstrating omega-3-induced improvements in skeletal muscle contraction efficiency in rats (Peoples & McLennan, 2010), lower steady-state submaximal whole body oxygen consumption in exercising humans (Peoples *et al.* 2008), potential fuel shifts during exercise in humans (i.e. strong trends for increased fat oxidation and glucose/glycogen sparing) (Delarue *et al.* 2003) and possibly the improvements seen in cardiac contractile function (Pepe & McLennan, 2002). However, in the current study we have not determined rates of ATP synthesis directly, and any beneficial consequence of increasing ADP sensitivity is based on the assumption of unaltered ATP/oxygen coupling. While rates of 'leak respiration' (absence of ADP) were not altered in the current study, uncoupling mitochondria could also be speculated to increase the apparent ADP sensitivity. However, a general uncoupling of mitochondria would also be expected to increase the V_{max} of all conditions and alter the apparent K_m for pyruvate, which did not occur. In addition, uncoupling mitochondria would be expected to decrease the capacity for mitochondrial ROS emission, although in the current study we observed an increased ROS emission. Therefore, the alteration in ADP sensitivity appears to be specific to the ADP protocols, and therefore may reflect an increased propensity for uncoupling during a metabolic challenge (i.e. the provision of ADP). However, omega-3 supplementation during a high-fat challenge in rodents does not alter the ADP/oxygen ratios in isolated mitochondria, challenging the notion that omega-3 supplementation increases the susceptibility of mitochondria to uncoupling (Lanza *et al.* 2013). Together, the current data suggest that ADP sensitivity is improved, probably through allosteric or covalent modifications of either ANT isoforms or ATP synthase.

The current data may also have implications to understanding the mechanisms of action by which omega-3 fish oils improve insulin sensitivity. The insulin-sensitizing effects of omega-3 fish oils have been proposed to involve remodelling of mitochondrial membrane phospholipid composition and a subsequent increase in substrate oxidation, particularly fatty acids (Borkman *et al.* 1993). However, it was recently reported in mice that the improvement of insulin sensitivity by omega-3 fish oils occurs independently of improvements in mitochondrial content or maximal respiratory function, challenging the 'mitochondria-centric' view of how omega-3 fatty acids

improve insulin sensitivity (Lanza *et al.* 2013). Therefore, the mechanistic basis for how omega-3 fish oils improve insulin sensitivity remains ambiguous. However, we have recently reported decreased ADP sensitivity within the skeletal muscle (Smith *et al.* 2013) of Zucker diabetic fatty rats, suggesting impaired ADP kinetics is a potential cause of insulin resistance. Therefore, it is tempting to speculate that an improvement in ADP sensitivity observed in the current study following fish oil supplementation represents a mechanism by which fish oils improve insulin sensitivity. The improvement in insulin sensitivity may also involve ROS-induced gene transcription of antioxidant enzymes, as recently proposed (Lanza *et al.* 2013). In the current study, mitochondrial ROS emission was increased, supporting this potential interpretation. It remains unknown mechanistically how omega-3 fish oils increase the propensity of mitochondria for ROS emission. However, the expected increase in mitochondrial membrane fluidity that would be expected following EPA and DHA incorporation into the mitochondrial membranes may create a 'tighter' interface between the phospholipid bilayer and integral membrane proteins, decreasing proton 'leak' and increasing mitochondrial ROS emission. While this mechanism is plausible, direct support for this supposition remains to be determined.

Clearly additional work examining the metabolic role of fish oil supplementation in various pathological conditions/models is warranted, especially in the context of ADP sensitivity. Supplementation with fish oils has long been recognized to have beneficial effects on the cardiovascular system, although the direct benefits to muscle bioenergetics have received less attention. The present findings strongly emphasize a role for omega-3s in reorganizing the composition of the mitochondrial membrane, and through an unknown mechanism, promoting beneficial adaptations to situations where ADP is progressively increased.

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Additional information

Competing interests

None declared.

Author Contributions

E.A.F.H., S.P., C.G., J.W., L.L.S. and G.P.H. designed experiments, performed experiments, analysed and interpreted data, and wrote the manuscript. K.M., A.C. and G.J.F. performed experiments, interpreted data and edited the manuscript.

Funding

This work was funded by The Natural Sciences and Engineering Research Council of Canada (NSERC; G.P.H., L.L.S.) and an operating grant to A.C. (N N401 292739). Infrastructure was purchased with the assistance of the Canadian Foundation for Innovation as well as the Ontario Research Fund. E.A.F.H. is supported by an NSERC graduate scholarship.

Acknowledgements

The authors would like to acknowledge the participants for their time and effort in completing this study.