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ARTICLE

Variable effects of 12 weeks of omega-3 supplementation on resting skeletal muscle metabolism[1]

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Abstract: Omega-3 supplementation has been purported to improve the function of several organs in the body, including reports of increased resting metabolic rate (RMR) and reliance on fat oxidation. However, the potential for omega-3s to modulate human skeletal muscle metabolism has received little attention. This study examined the effects of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) supplementation on whole-body RMR and the content of proteins involved in fat metabolism in human skeletal muscle. Recreationally active males supplemented with 3.0 g/day of EPA and DHA (n = 21) or olive oil (n = 9) for 12 weeks. Resting muscle biopsies were sampled in a subset of 10 subjects before (pre) and after (post) omega-3 supplementation. RMR significantly increased (5.3%, p = 0.040) following omega-3 supplementation (Pre, 1.33 ±0.05; Post, 1.40 ±0.04 kcal/min) with variable individual responses. When normalizing for body mass, this effect was lost (5.2%, p = 0.058). Omega-3s did not affect whole-body fat oxidation, and olive oil did not alter any parameter assessed. Omega-3 supplementation did not affect whole muscle, sarcolemmal, or mitochondrial FAT/CD36, FABPpm, FATP1 or FATP4 contents or mitochondrial electron chain and PDH proteins, but did increase the long form of UCP3 by 11%. In conclusion, supplementation with a high dose of omega-3s for 12 weeks increased RMR in a small and variable manner in a group of healthy young men. Omega-3 supplementation also had no effect on several proteins involved in skeletal muscle fat metabolism and did not cause mitochondrial biogenesis.

Key words: omega-3, eicosapentaenoic acid, docosahexaenoic acid, skeletal muscle, substrate oxidation.

Résumé : La supplémentation en oméga-3 améliorerait, semble-t-il, le fonctionnement de plusieurs organes, augmenterait, selon des rapports, le métabolisme de repos (« RMR ») et le recours à l'oxydation des graisses. Toutefois, il y a peu d'études sur le rôle potentiel des oméga-3 dans la modulation du métabolisme du muscle squelettique humain. Cette étude examine les effets de la supplémentation en acide eicosapentanoïque (AEP) et en acide docosahexanoïque (ADH) sur le RMR global de l'organisme et sur le contenu en protéines impliquées dans le métabolisme des graisses dans le muscle squelettique humain. On demande à des hommes actifs par loisir de prendre à raison de 3,0 g/jour durant 12 semaines des suppléments d'AEP et de ADH (n = 21) ou de l'huile d'olive (n = 9). On prélève des échantillons de tissu musculaire au repos chez un sous-ensemble de 10 sujets avant (« Pre ») et après (« Post ») la supplémentation en oméga-3. Le RMR augmente significativement (5,3 %, p = 0,040) après la supplémentation en oméga-3 (Pré, 1,33 ± 0,05; Post, 1,40 ± 0,04 kcal/min), mais on note des différences interindividuelles. En normalisant les données en fonction de la masse corporelle, l'effet s'annule (5,2 %, p = 0,058). Les oméga-3 ne changent pas l'oxydation globale des graisses dans l'organisme et l'huile d'olive ne modifie aucune des variables évaluées. La supplémentation en oméga-3 ne modifie pas le contenu en protéines de FAT/CD36, FABPpm, FATP1 ou FATP4 dans le muscle en entier, le sarcolemme ou la mitochondrie, la chaîne de transport des électrons et la PDH, mais augmente de 11% la forme allongée de l'UCP3. En conclusion, la supplémentation à forte dose d'oméga-3 durant 12 semaines suscite une légère augmentation variable du RMR chez un groupe de jeunes hommes en bonne santé. La supplémentation en oméga-3 n'a pas d'effet sur plusieurs protéines impliquées dans le métabolisme des gras dans le muscle squelettique et ne suscite pas de biogenèse dans la mitochondrie. [Traduit par la Rédaction]

Mots-clés : oméga-3, acide eicosapentanoïque, acide docosahexanoïque, muscle squelettique, oxydation des substrats.

Introduction

A growing body of evidence suggests that omega-3 fatty acids, specifically eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), possess the potential to affect energy metabolism. Early studies observed a strong positive correlation between higher metabolic activity and a higher degree of cell membrane unsaturation across different species (Hulbert and Else 1999). This suggests that the metabolic rate could be raised by increasing the consumption of unsaturated fatty acids and their incorporation into membranes. Further evidence suggests that EPA and DHA supplementation may also promote a shift in whole-body substrate utilization to increased fat use and lead to reductions in body fat (Buckley and Howe 2010). In support, omega-3 supplementation has been shown to increase the expression of genes associated with lipid oxidation and decrease the expression of lipogenic genes in the liver, adipose tissue, and to a certain extent, skeletal muscle of rodents (Price et al. 2000; Lapillonne et al. 2004; Sampath and Ntambi 2004).

While it is tempting to conclude that omega-3 supplementation increases RMR and fat oxidation, the data in humans are limited and inconclusive. Initial observations by Couet et al. (1997) showed that 3 weeks of fish oil supplementation (6 g/day, 1.1 g EPA and 0.7 g DHA) in healthy, sedentary young adults (n = 6) significantly increased resting metabolic rate (RMR) and basal lipid oxidation, with no changes in carbohydrate (CHO) oxidation

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compared with supplementing with no fish oil. However, subjects supplementing with fish oil experienced a nonsignificant 0.2-kg increase in fat-free mass (FFM), and the increase in RMR was no longer present when normalized to FFM. Bortolotti and colleagues (2007) gave young healthy sedentary males (n = 8) EPA + DHA (1.1 + 0.7 g/day) for 15 days and reported no increase in energy expenditure but increased fat and decreased CHO oxidation. However, the RMR measurements were made after feeding subjects a high-CHO meal (80% of total energy) prior to testing, whereas RMR is typically measured following an overnight fast. More recently, Noreen et al. (2010) reported that supplementation with 1.6 g EPA and 0.8 g DHA per day for 6 weeks in healthy, young active men and women (n = 12) did not change RMR or substrate oxidation despite the longer supplementation period. Finally, Kratz et al. (2009) examined whole-body energy metabolism in overweight and moderately obese men and women (n = 13) and also found no effect of a combination of EPA, DHA, and alpha-linolenic acid (3.6% of total energy) on RMR and respiratory exchange ratio (RER) compared with controls, even with a 14-week supplementation period.

It is difficult to draw definitive conclusions on the effect of EPA and DHA on RMR and substrate metabolism in humans based on this limited research, as study subject characteristics and numbers, dosages, and supplementation durations were inconsistent.

Skeletal muscle is a large organ and a significant site of glucose and lipid metabolism. It also represents a significant proportion (~20%) of the whole-body resting energy expenditure (Zurlo et al. 1990). Despite this, little attention has been directed to the potential for EPA and DHA to modulate skeletal muscle energy metabolism. Omega-3s are potent ligand activators of peroxisome proliferator-activated receptors (PPARs), a family of transcription factors widely known for their role in energy homeostasis through the regulation of genes responsible for glucose and lipid metabolism (Kota et al. 2005). EPA and DHA supplementation has been shown to increase the skeletal muscle expression of the PPAR targets fatty acid translocase (FAT/CD36) (Aas et al. 2006), cytoplasmic fatty acid binding protein (FABPc) (Clavel et al. 2002), and uncoupling protein-3 (UCP3) (Baillie et al. 1999) in cell and rodent muscle models. In addition, increased omega-3 exposure has been implicated in increasing the oxidative potential of isolated muscle cells and rodent skeletal muscle through mitochondrial biogenesis in some (Totland et al. 2000; Vaughan et al. 2012), but not all (Smith et al. 2010) studies. Recently, it has been shown that omega-3s can also increase gene expression in human skeletal muscle (Jans et al. 2012), but we are unaware of any work demonstrating that these changes translate into protein changes.

The first aim of this study was to re-examine the effects of omega-3 supplementation on whole body resting energy expenditure and substrate oxidation by utilizing a high dosage, long supplementation period, and large sample size. Given these parameters, we predicted that RMR and fat oxidation would increase as a result of omega-3 supplementation and not change in the placebo group. A second aim was to determine whether omega-3 supplementation would increase the content of PPAR target proteins involved in the regulation of fat transport across the muscle and mitochondrial membranes (FAT/CD36; plasma membrane fatty acid binding protein, FABPpm; and fatty acid transport proteins 1 and 4, FATP1, FATP4) and proteins representative of mitochondrial biogenesis in human skeletal muscle.

Materials and methods

Subjects

Thirty-six healthy, recreationally active males volunteered to participate in this study. Written informed consent was received from each subject following a detailed explanation of the experimental protocol and any associated risks. Subjects were screened to ensure they were in good health, were not currently taking omega-3 supplements, had no previous history of omega-3 supplementation, and were not currently or had previously consumed a diet high in omega-3s. Subjects were instructed to maintain consistent diet and exercise habits throughout the study, and physical activity records, 3-day dietary records, and body mass were obtained at the beginning, midway point, and end of the supplementation period. The study was approved by the University of Guelph and McMaster University Research Ethics Boards.

Table 1. Subject characteristics before (Pre) and after (Post) 12 weeks of omega-3 or placebo supplementation.

	Omega-3		Placebo	
	Pre	Post	Pre	Post
Age (y)	22.7±0.8		20.6±0.4	
Height (cm)	182.8±1.5		179.9±0.8	
Body mass (kg)	82.1±2.2	82.5±2.3	79.0±3.2	78.2±3.0
Physical activity (min/wk)	209.6±18.2	190.9±21.5	165.6±25.1	188.9±47.5
Diet (Cal)	2295±127	2075±144*	2320±138	2257±141

Note: Values are means ± SE (omega-3, n = 21; placebo, n = 9).
*Significantly different from Pre.

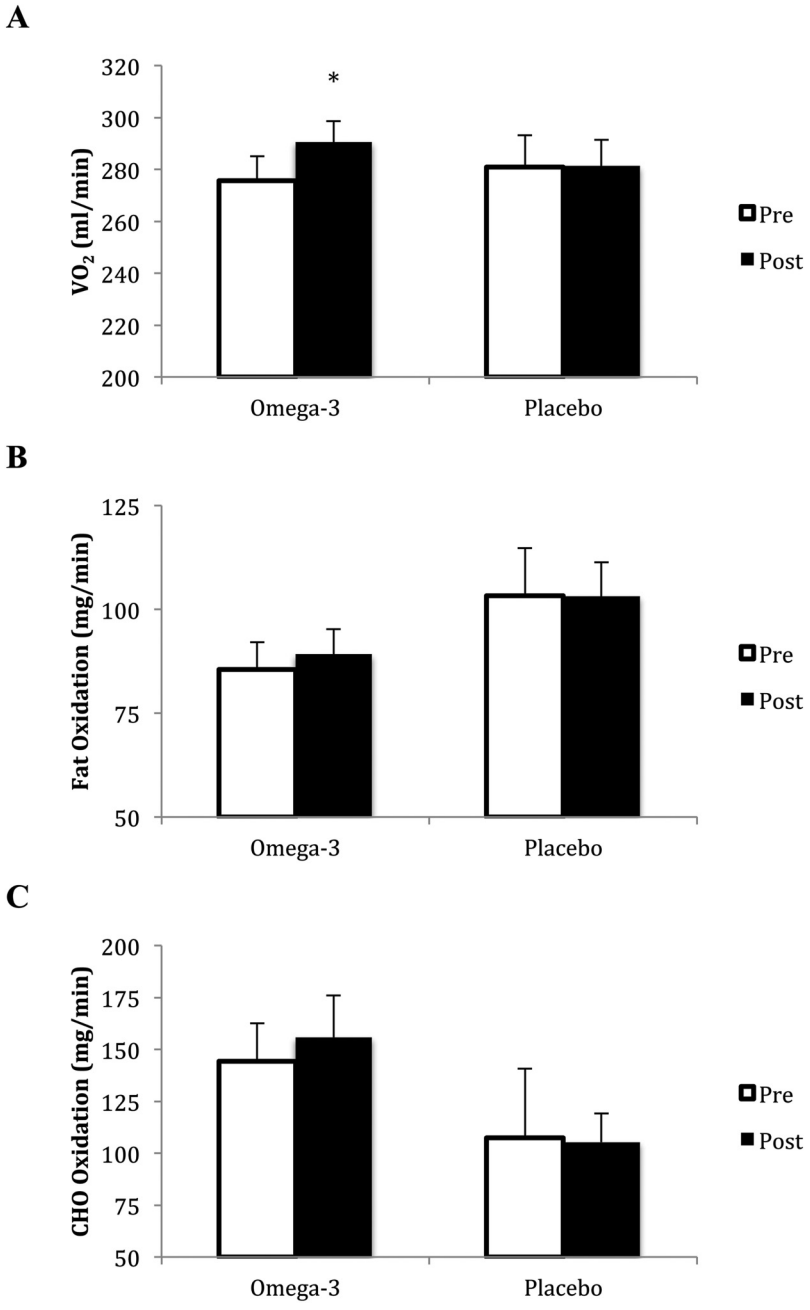
Study design

Subjects were randomly assigned in a single-blind manner to supplement with omega-3 or placebo capsules prior to testing. Subjects reported to the laboratory following an overnight fast for measurements of resting metabolic rate. Following this trial, a blood sample was taken and later analyzed for serum omega-3 content. In addition, 10 subjects in the omega-3 group received resting skeletal muscle biopsies. Subjects were asked to refrain from any physical activity, alcohol, and caffeine consumption 24 h prior to each trial, and to consume a balanced diet (~50% of energy (E%) from CHO, ~30 E% from fat, and ~20 E% from protein) the day before each trial. Diet records were obtained from the day before each presupplementation trial, and subjects were instructed to follow the same diet the day before each postsupplementation trial. Following baseline measurements, supplementation occurred for 12 weeks as follows:

1. Omega-3 group (n = 21): Twenty-four subjects were randomly assigned to the omega-3 group, with a total of 21 completing the study. Subjects supplemented with 5 capsules of Omega-3 Complete (1000 mg per capsule; Jamieson Laboratories Ltd., Windsor, Ont., Canada) per day. Each capsule provided 400 mg of EPA and 200 mg of DHA in triglyceride (TG) form, for a total of 2 g EPA and 1 g DHA per day. EPA and DHA in the TG form were chosen for this study as the short- and long-term bioavailability has recently been shown to be significantly greater than the common ethyl ester form found in most supplements (Dyerberg et al. 2010; Neubronner et al. 2011). Subjects were instructed to take 2 capsules in the morning with breakfast and 3 with evening dinner.
2. Placebo (n = 9): Twelve subjects were randomly assigned to the placebo group, with a total of 9 completing the study. Subjects supplemented with 3 capsules of Swanson EFAs Certified Organic Extra Virgin Olive Oil, Cold-Pressed (1000 mg per capsule; Swanson Health Products, Fargo, N.D., USA) per day. Subjects were instructed to take 1 capsule in the morning with breakfast and 2 with evening dinner. Subjects were given only 3 capsules of olive oil to minimize any effects this oil (high in monounsaturated fatty acids) may have on the measurements of the study.

To promote supplement compliance, subjects were only given 2 weeks of capsules at a time. Written and oral reminders were also given periodically to ensure diet and exercise practices were maintained consistently throughout the study. Following the 12-week supplementation period, all testing was repeated. Instructions for postsupplementation trials were the same as given for the presupplementation trials.

Fig. 1. Mean resting whole-body oxygen consumption (VO_2) (A), fat oxidation (B), and carbohydrate (CHO) oxidation (C) before (Pre) and after (Post) 12 weeks of omega-3 or placebo supplementation. Values are means ± SE (omega-3, $n = 21$; placebo, $n = 9$). *, Significantly different from Pre ($p < 0.05$).



Resting metabolic rate

Subjects arrived at the laboratory following an overnight fast (10–12 h). They were instructed to lie supine on a bed for ~30 min in a quiet, darkened room. The room temperature was always between 20–22 °C and the subjects wore their own clothes and exactly the same clothes for the final measures. Subjects had mouthpieces in and nose plugs on from minutes 10–30 and respiratory measurements were collected during the last ~17 min using the mixing chamber mode of the metabolic cart (AEI MOXUS II Metabolic System, Pittsburgh, Pa., USA). The last 15 min of data were analyzed for resting oxygen consumption ($\dot{V}O_2$) and carbon dioxide production ($\dot{V}CO_2$). Heart rate was recorded every 5 min using a Polar Heart Rate watch (Polar Electro, Finland). Following the respiratory measurements, a venous blood sample was taken for all subjects.

Calculations

RER was calculated as $\dot{V}CO_2/\dot{V}O_2$. Whole-body resting fat oxidation, CHO oxidation, and energy expenditure were calculated using the following equations (Péronnet and Massicotte 1991):

$$\text{Fat oxidation} = (1.695 \times \dot{V}O_2) - (1.701 \times \dot{V}CO_2)$$

where fat oxidation is in g/min and $\dot{V}O_2$ and $\dot{V}CO_2$ are in L/min.

$$\text{CHO oxidation} = (4.585 \times \dot{V}CO_2) - (3.226 \times \dot{V}O_2)$$

where CHO oxidation is in g/min and $\dot{V}CO_2$ and $\dot{V}O_2$ are in L/min.

$$\text{Energy expenditure} = \dot{V}O_2 \times \text{RER caloric equivalent} \times \text{time}$$

where energy expenditure is in kcal, $\dot{V}O_2$ is in L/min, RER caloric equivalent is in kcal/L, and time is in minutes.

Muscle biopsies

Two to 4 resting muscle biopsies (total ~400–500 mg) were obtained under local anesthesia (2% lidocaine without epinephrine) from the vastus lateralis muscle, using the percutaneous needle biopsy technique described by Bergstrom 1975. Pre- and postsupplementation biopsies were obtained from opposite legs. One muscle aliquot (~200–250 mg) was used to isolate the sarcolemmal membrane by preparing giant sarcolemmal vesicles and a second aliquot (~200–250 mg) was used for the isolation of mitochondria.

Preparation of giant sarcolemmal vesicles

Giant sarcolemmal vesicles (GSV) were generated as described previously (Bonen et al. 2000; Talanian et al. 2010). Briefly, the tissue was cut into thin layers that were ~1–3 mm thick and incubated for 1 h at 34 °C in 140 mmol/L KCl/10 mmol/L MOPS (pH 7.4), 1 mL of collagenase (type VII, 150 units/mL), and 30 μg/mL aprotinin in a shaking water bath. Following incubation the sample was filtered through cheesecloth and the supernatant fraction was collected. The remaining tissue was washed with KCl MOPS and 10 mmol/L EDTA, resulting in a second supernatant fraction. The 2 supernatant fractions were pooled, and Percoll (G.E. Healthcare, Baie d'Urfe, Que., Canada), KCl, and aprotinin were added to final concentrations of 3.5% (*v*/*v*), 20 mmol/L, and 10 μg/mL, respectively. The resulting suspension was placed at the bottom of a density gradient consisting of a 3-mL middle layer of 4% Nycodenz (*w*/*v*) and a 1-mL upper layer of KCl/MOPS. The sample was then centrifuged at 60*g* for 45 min at room temperature. The vesicles were then harvested from the interface of the upper and middle solutions, diluted in KCl/MOPS, and recentrifuged at 12 000*g* for 5 min. The resultant vesicle pellet was resuspended in KCl/MOPS and stored at −80 °C for Western blot analyses. The remaining homogenate was used for whole-muscle homogenate Western blot analyses.

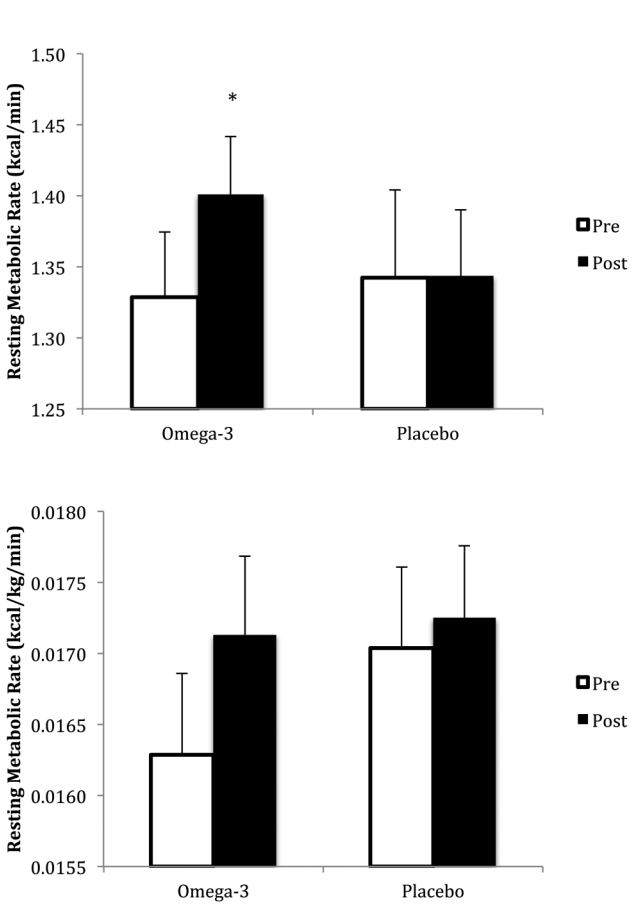
Mitochondrial isolation

Intact, pooled mitochondria (containing both intermyofibrillar (IMF) and subsarcolemmal (SS) fractions) were isolated as described previously (Holloway et al. 2007; Talanian et al. 2010). Briefly, fresh muscle was homogenized and centrifuged at 800*g* for 10 min to separate SS and IMF mitochondria. IMF mitochondria were treated with protease (Subtilisin A; Sigma, St. Louis, Mo., USA) for exactly 5 min and centrifuged to remove the myofibrils. IMF and SS fractions were then combined and centrifuged at 10 000*g* twice for 10 min. Samples were purified further using a Percoll gradient. Samples were centrifuged at 20 000*g* for 1 h and the mitochondrial layer was removed. Additional centrifugation at 20 000*g* for 5 h removed the Percoll from the sample. Samples were frozen at −80 °C for analysis by Western blot.

Western blot analysis

Buffer containing Triton X-100 (1% *v*/*v*) Tris-HCl (50 mmol/L), EDTA (1 mmol/L), EGTA (1 mmol/L), NaF (50 mmol/L), sodium β-glycerol phosphate (10 mmol/L), sodium pyrophosphate (5 mmol/L), DTT (2 mmol/L), sodium orthovanadate (1 mmol/L), phenylmethylsulfonyl fluoride (1 mmol/L), aprotinin (10 μg/mL), leupeptin (10 μg/mL), and pepstatin A (10 μg/mL) was added to 50–60 mg of whole muscle, and samples were homogenized (Fast Prep 24 Lysing Matrix D tubes) for 60 s. Following homogenization, samples were centrifuged at 1500*g* for 15 min at 4 °C, and the resulting supernatant was collected.

Fig. 2. Absolute mean resting metabolic rate (A) and normalized to body mass (B) before (Pre) and after (Post) 12 weeks of omega-3 or placebo supplementation. Values are means ± SE (omega-3, $n = 21$; placebo, $n = 9$). *, Significantly different from Pre ($p < 0.05$).



Isolated mitochondrial, whole muscle, and GSV samples were analyzed for total protein content (BCA protein assay), and 5 μg, 20 μg, and 5 μg of protein was loaded for Western blotting, respectively, with the exception of UCP3, where 50 μg of isolated mitochondrial sample was loaded.

Samples were separated by electrophoresis on an 8%, 10%, or 12% SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane. Membranes were blocked with 7.5% bovine serum albumin or 5% nonfat milk, and immunoblotted for 4 h or overnight with primary antibodies at 4 °C. Gel, block, and immunoblot conditions were dependent on the specific protein being measured. The monoclonal antibody MO-25 and antisera against FABPpm were used to detect FAT/CD36 and FABPpm, respectively (gifts from N.N. Tandon and J. Calles-Escandon, respectively). Commercially available antibodies were used to detect FATP1 and FATP4 (Santa Cruz Biotechnology, Dallas, Tex., USA), PDH subunit E1α (Molecular Probes, Eugene, Ore., USA), UCP3 (Millipore, Billerica, Mass., USA), total OXPHOS (Abcam, Toronto, Ont., Canada), sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA) (Sigma–Aldrich, Oakville, Ont., Canada), and caveolin-1 (BD Biosciences, Mississauga, Ont., Canada). Membranes were incubated at room temperature for 1 h with the corresponding secondary antibody, and proteins were detected with enhanced chemiluminescence (Perkin Elmer Life Science, Boston, Mass., USA). Blots were subsequently quantified by densitometry using Alpha Innotech software. Blots were stained with Ponceau-S to ensure equal loading of protein.

Fig. 3. Mean resting metabolic (RMR) rate for each participant normalized to body mass, before (Pre) and after (Post) 12 weeks of omega-3 or placebo supplementation. Participants in each group are arranged from lowest to highest baseline RMR. A significant negative correlation existed between baseline RMR and the increase in RMR following omega-3 supplementation ($r = -0.49$, $p = 0.02$).

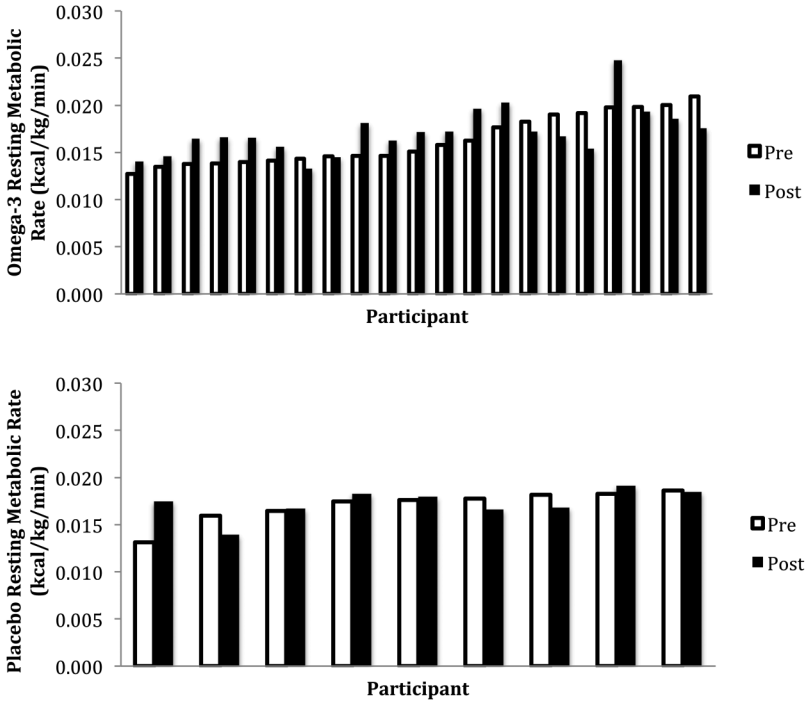
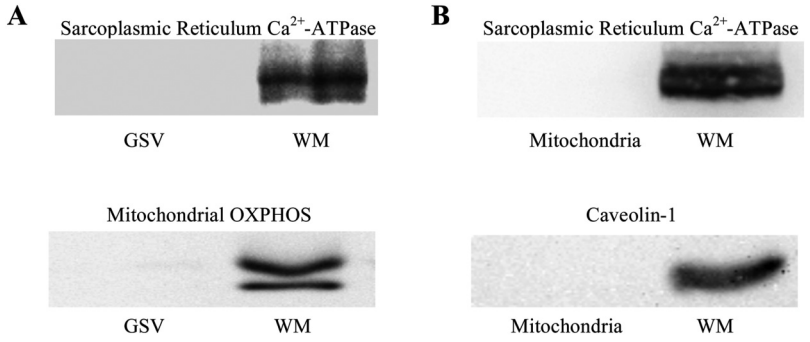


Fig. 4. Representative Western blots performed on giant sarcolemmal vesicles (A) and isolated mitochondria (B) from human skeletal muscle. Whole-muscle homogenate from human skeletal muscle was used as a positive control. GSV, giant sarcolemmal vesicles; WM, whole muscle.



Blood analyses

Fasted blood samples were collected in serum tubes and analyzed for EPA and DHA content as described previously (Zulyniak et al. 2013).

Statistical analysis

All data are presented as means ± SE. Differences between pre- and postsupplementation measurements were analyzed with a paired, one-tailed t test. Statistical significance was accepted at $p < 0.05$.

Results

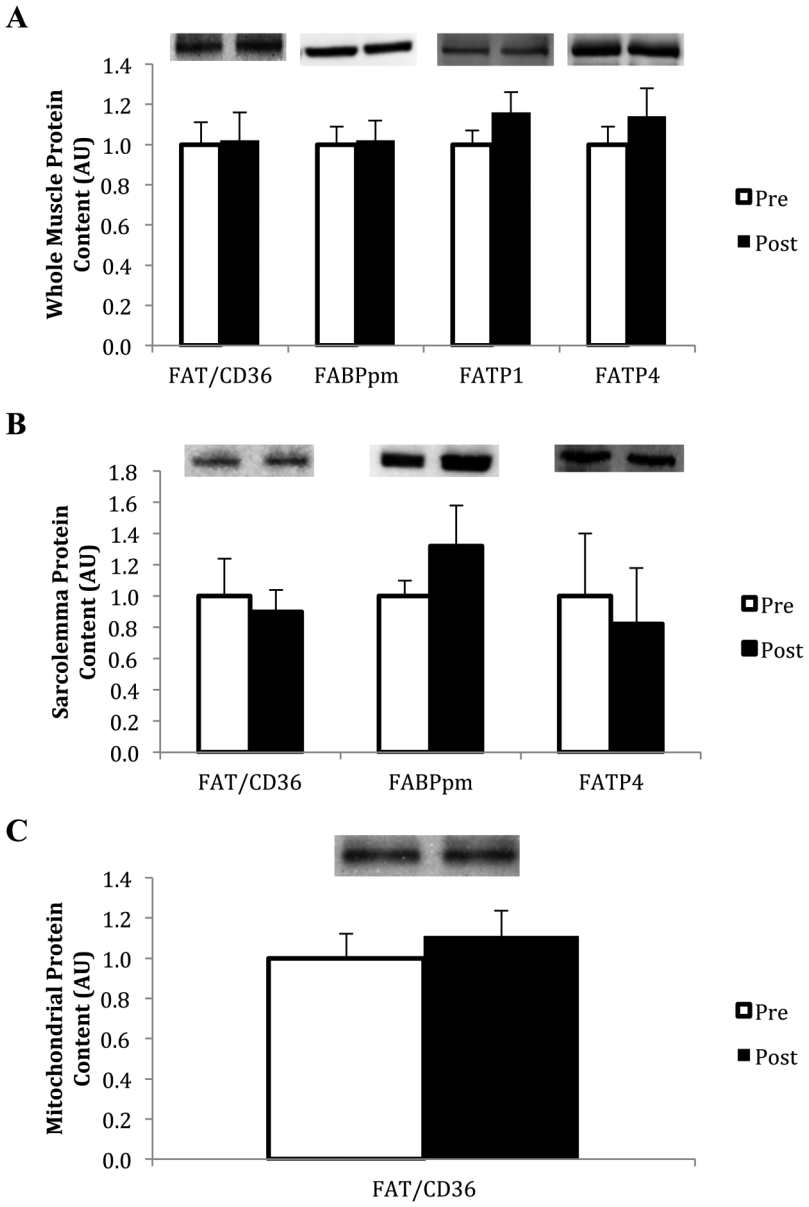
Subject characteristics

Body mass, weekly exercise, and daily energy intake did not change in either group over the 12-week supplementation period, with the exception of a decrease in total kcal in the omega-3 group, calculated from 3-day diet records (Table 1). The percent composition of the diets was consistent within and between groups (~53% CHO, 21% fat, 26% protein). Serum fatty acid analysis demonstrated significant increases in the relative percentage of EPA (Pre, 0.58 ± 0.04; Post, 3.68 ± 0.23) and DHA (Pre, 1.45 ± 0.06; Post, 3.51 ± 0.15) in the omega-3 subjects, indicating supplementation compliance. Serum EPA (Pre, 0.44 ± 0.05; Post, 0.52 ± 0.07) and DHA (Pre, 1.00 ± 0.12; Post, 1.01 ± 0.10) did not change in the placebo group. Of the subjects in the omega-3 group, 73% of the subjects were not aware that they were taking omega-3s at the end of the study.

Resting whole-body energy expenditure and substrate oxidation

The average resting $\dot{V}O_2$ significantly increased by 5.4% (Pre, 275.7 ± 9.4; Post, 290.5 ± 8.0 mL/min) (Fig. 1A) and $\dot{V}CO_2$ significantly increased by 5.8% (Pre, 224.8 ± 9.1; Post, 237.9 ± 8.9 mL/min) following omega-3 supplementation. No changes occurred in the $\dot{V}O_2$ (Pre, 280.8 ± 12.2; Post, 281.3 ± 10.0 mL/min) (Fig. 1A) or $\dot{V}CO_2$ (Pre, 219.2 ± 14.5; Post, 219.6 ± 7.9 mL/min) of the placebo group. No changes in ventilation occurred with omega-3 (Pre, 8.6 ± 0.4; Post, 8.4 ± 0.4 L/min) or placebo (Pre, 8.4 + 0.5; Post, 8.6 + 0.3 L/min) supplementation. Resting RER did not change in either the omega-3 (Pre, 0.82 ± 0.01; Post, 0.82 ± 0.01) or placebo (Pre, 0.78 ± 0.03; Post, 0.78 ± 0.01) groups. The resting fat oxidation rate was

Fig. 5. Human skeletal muscle protein content of fatty acid transporters in whole-muscle homogenate (A, $n = 10$), giant sarcolemmal vesicles (B, $n = 9$), and isolated mitochondria (C, $n = 10$) before (Pre) and after (Post) 12 weeks omega-3 supplementation. Values are means ± SE in arbitrary units (AUs). FABPpm, plasma membrane fatty acid binding protein; FAT/CD36, fatty acid translocase; FATP1, FATP4, fatty acid transport proteins 1 and 4.



not significantly changed in the omega-3 (Pre, 85.6 ± 6.5; Post, 89.2 ± 6.0 mg/min) or placebo groups (Pre, 103.3 ± 11.6; Post, 103.1 ± 8.2 mg/min) (Fig. 1B) following supplementation. Similarly, the CHO oxidation rate did not significantly change in the omega-3 (Pre, 144.3 ± 18.4; Post, 155.8 ± 20.2 mg/min) or placebo (Pre, 107.4 ± 33.3; Post, 105.3 ± 13.8 mg/min) groups (Fig. 1C).

RMR significantly increased by 5.3% (Pre, 1.33 ± 0.05; Post, 1.40 ± 0.04 kcal/min) following omega-3 supplementation, with no changes occurring in the placebo group (Pre, 1.34 ± 0.06; Post, 1.34 ± 0.05 kcal/min) (Fig. 2A). Normalizing to body mass resulted in a similar increase of 5.2% in the omega-3 group, although the increase was not statistically significant ($p = 0.058$), with no change in the placebo (Fig. 2B). A two-way, repeated measures ANOVA with the 9 placebo subjects and the first 9 omega-3 subjects also demonstrated no significant main effect for treatment ($p = 0.196$) or time ($p = 0.707$) and no interaction ($p = 0.939$).

Individual subject changes highlight the significant variability that existed between participants (range = –20% to + 25%, Fig. 3). For example, if a change > 5% is considered to be a meaningful increase in RMR, a change < 5% is considered to be a decrease, and a ±5% difference is considered to be no change in RMR, 13 subjects experienced an increase in RMR (range = 8%–25%) following omega-3 supplementation, 6 subjects decreased (range = 6%–20%), and 2 subjects did not change. There was a significant negative correlation between baseline RMR and the absolute increase in RMR following omega-3 supplementation ($r = -0.49$, $p = 0.02$), where the lower the baseline RMR the more likely that omega-3 supplementation would increase RMR.

There was no change in resting heart rate in the omega-3 (Pre, 62.3 ± 2.4; Post, 60.3 ± 1.6 beats/min) and placebo (Pre, 65.1 ± 3.0; Post, 60.5 ± 3.0 beats/min) groups.

Skeletal muscle protein content

Western blotting demonstrated that sarcolemmal and mitochondrial membrane isolation procedures were free of contamination from other cell fractions (Fig. 4A, 4B).

Omega-3 supplementation did not change the whole-muscle protein content of FAT/CD36, FABPpm, FATP1, or FATP4 (Fig. 5A), the sarcolemmal protein content of FAT/CD36, FABPpm, or FATP4 (Fig. 5B), or the mitochondrial protein content of FAT/CD36 (Fig. 5C). Due to tissue limitations, sarcolemmal FATP1 was not measured.

Total mitochondrial UCP3 content was not affected by omega-3 supplementation (Fig. 6). Long and short forms of UCP3 were also detected, with a significant increase in the long form (11%, $p < 0.05$) and no change in the short form.

There was no change in mitochondrial content following supplementation, as indicated by the protein content of specific subunits of the electron transport chain complexes I–V and the E1α subunit of PDH (Fig. 7).

Discussion

The present study demonstrated that supplementing with 2 g of EPA and 1 g DHA per day for 12 weeks significantly increased whole-body $\dot{V}O_2$ and RMR, and the protein content of the long form of skeletal muscle UCP3 (11%) of young healthy males. Supplementation did not affect resting fat oxidation or increase the content of whole muscle, sarcolemmal, or mitochondrial fatty acid transport proteins or the mitochondrial content of human skeletal muscle. It is important to note that the increase in RMR was small and variable, and the significance of the finding was lost when normalized to body mass. In spite of utilizing a high omega-3 dose, large sample size, and long supplementation period, the magnitude of the group increase in RMR was 5.3%, with a range of –20% to + 25%.

Resting metabolic rate

Couet et al. 1997 suggested that omega-3 fatty acids have the ability to increase whole-body RMR in humans, but subsequent studies have been unable to reproduce these findings (Bortolotti et al. 2007; Kratz et al. 2009; Noreen et al. 2010). Inconsistencies in populations, dosage, supplementation period, and study design have made it difficult to make definitive conclusions. The present study used a high dose and long supplementation period and reported increased resting $\dot{V}O_2$ and RMR with omega-3 supplementation. The average RMR increase translates into a daily increase of only ~100 kcal. The increase in RMR reported by Couet et al. 1997 was even smaller (~65 kcal/day) and it is not immediately clear how biologically significant these small changes are. The present study confirms that the effect of omega-3 supplementation is not large, thereby also contributing to the lack of consistency in significant findings between studies.

Omega-3 supplementation may induce biologically significant changes in some but not all individuals and the possibility of responders and nonresponders has recently been discussed (Nording et al. 2013). Of the 13 subjects who experienced an increase in RMR based on the arbitrary criteria we chose (>5% increase, repeatability in our laboratory was 4%), 12 had a daily increase of ~175 kcal or greater following omega-3 supplementation, and 7 had a daily increase greater than 250 kcal. This represents a large biological effect in a subset of subjects that is not apparent when examining the group mean. A low baseline RMR predicted a larger effect of omega-3s, although the prediction was weak ($r^2 = 0.24$). In addition, even if the threshold for determining an effect of omega-3s was moved to 10% versus 5%, it did not alter the individual variability data significantly. There were still 11 subjects classified as having an effect, with the 2 participants being lost having an effect size of 8% and 9%.

When normalized to body mass, the increase in RMR was no longer statistically significant on a group basis. This has also been reported when normalized to FFM (Couet et al. 1997). Omega-3s have been documented to increase FFM by 0.2–0.5 kg and decrease fat mass (Couet et al. 1997; Noreen et al. 2010), as well as estimated muscle mass gains (~0.4 kg) (Smith et al. 2011). This represents a potential mechanism for the increase in RMR in the present study. Lean body mass is a main determinant of RMR (Illner et al. 2000) and increases in RMR were related to increases in lean mass (Pratley et al. 1994; Lemmer et al. 2001). It is difficult to measure small changes in FFM and we did not measure body composition changes in the present study. However, based on previous evidence it is possible that the small body mass increase (0.4 kg) that occurred in the omega-3 group was due to changes in FFM and not fat mass. Future work would benefit from sensitive and accurate measures of FFM pre- and post-omega-3 supplementation.

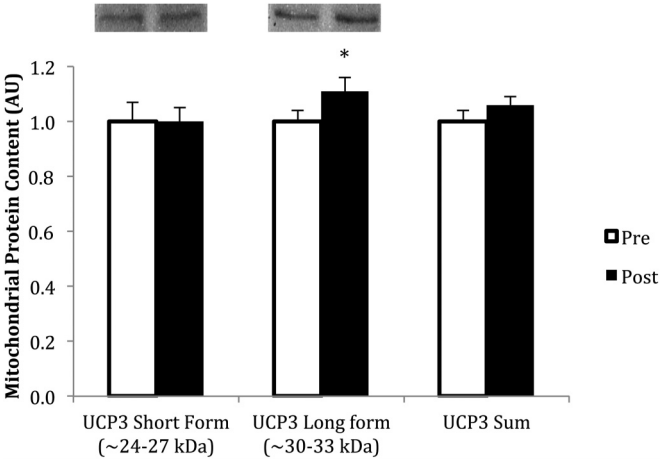


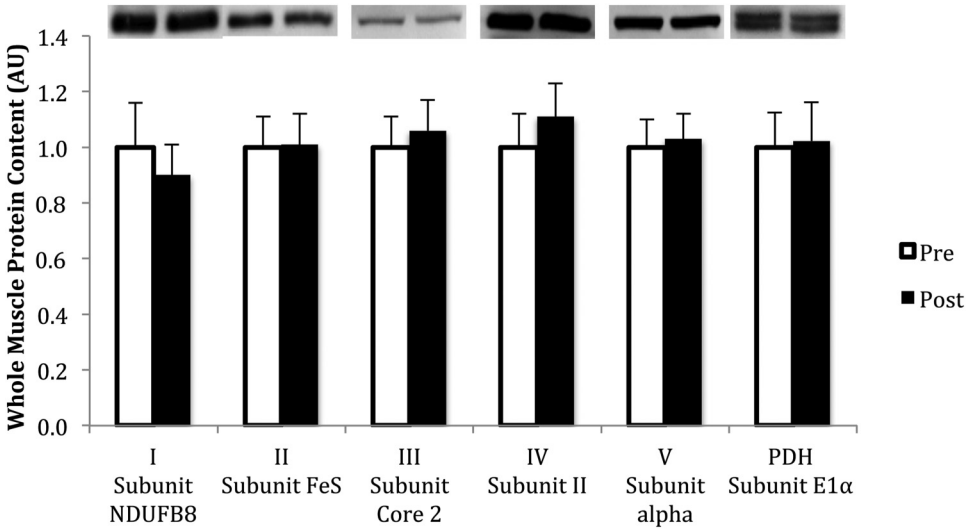
Fig. 6. Human skeletal muscle protein content of uncoupling protein 3 (UCP3) in isolated mitochondria before (Pre) and after (Post) 12 weeks of omega-3 supplementation. Values are means ± SE in arbitrary units (AUs) ($n = 9$). *, Significantly different from Pre ($p < 0.05$). UCP3, uncoupling protein-3.

Cellular mechanisms for increased energy expenditure with omega-3 supplementation

The incorporation of omega-3 fatty acids into cell membranes is suggested to alter the structural and compositional properties of membranes, which may influence membrane protein activity and function (Spector and Yorek 1985; McIntosh and Simon 2006). We recently reported significant incorporation of EPA and DHA into mitochondrial membranes and DHA into sarcolemmal membranes of human skeletal muscle following omega-3 supplementation (Herbst et al. 2014; Gerling et al. 2014). Although the functional effects of these membrane changes have not been elucidated, several theories have been proposed to support an increase in $\dot{V}O_2$ and RMR following omega-3 supplementation. The pacemaker theory of metabolism proposes that increased membrane unsaturation is associated with a higher metabolic rate (Hulbert and Else 1999). The theory postulates that the physical properties of unsaturated membranes results in elevated activity of some membrane proteins or membrane-associated processes. For example, the activities of Na^+/K^+-ATPase, Ca^{2+}-ATPase, and maintenance of the mitochondrial proton gradient, all of which utilize ATP, are positively correlated with membrane DHA content (Hulbert et al. 2005). This subsequently results in increased rates of ATP consumption, and a higher metabolic rate of the whole organism (Hulbert 2007).

A second theory suggests that incorporation of omega-3s into mitochondrial membranes (higher unsaturation) elevates energy expenditure because of an increase in proton leak. Protons cross the inner mitochondrial membrane independent of complex V and the energy normally coupled to ATP production is dissipated, causing a lower energy yield per NADH. It has been shown that a

Fig. 7. Human skeletal muscle protein content of electron transport chain complexes I–V and PDH subunit E1α in whole-muscle homogenate before (Pre) and after (Post) 12 weeks of omega-3 supplementation. Values are means ± SE in arbitrary units (AUs) (n = 10).



higher membrane content of DHA in rodent liver mitochondria was positively correlated to an increase in proton leak (Hulbert et al. 2005). The small increase in UCP3 (11%) reported in this study may contribute to increased proton leak, suggesting that omega-3 supplementation may increase skeletal muscle oxygen consumption and metabolic rate in this manner. However, the weak association between the change in RMR (kcal/min) and increase in UCP3 content (r = 0.25, p = 0.51) suggests this is not the case. In addition, state 4 respiration was not different in human skeletal muscle mitochondria and mitochondrial reactive oxygen species were higher, suggesting that mitochondria are not leaky following omega-3 supplementation (Herbst et al. 2014).

Fat metabolism

Studies utilizing short supplementation periods (2–3 weeks) reported that omega-3 fatty acids increased the reliance on fat as a fuel (Couet et al. 1997; Bortolotti et al. 2007). However, the present study did not observe changes in whole-body fat oxidation following 12 weeks of omega-3 supplementation, which is consistent with other reports using longer supplementation periods (Kratz et al. 2009; Noreen et al. 2010). Independent of these whole-body results, there is evidence for increased skeletal muscle fat metabolism in rodent skeletal muscle, which will be discussed below.

Omega-3 supplementation increased the gene expression of FAT/CD36 (Aas et al. 2006) and UCP3 (Baillie et al. 1999), and protein expression of FABPc (Clavel et al. 2002). Power and Newsholme 1997 also reported an increase in the maximal activity of CPTI and decreased sensitivity of CPTI to malonyl-CoA inhibition following omega-3 supplementation in rodent skeletal muscle. In addition, Holloway et al. 2012 isolated red and white skeletal muscle SS and IMF mitochondria from rodent hindlimbs and reported a significant positive correlation between the unsaturation index of mitochondria and palmitate oxidation in both muscles. However, in the present study, supplementation did not increase the content of whole muscle, sarcolemmal, or mitochondrial fatty acid transport proteins or the mitochondrial content of human skeletal muscle. These proteins were unaffected even though we recently demonstrated that omega-3 supplementation increased the incorporation of EPA and DHA into mitochondrial membranes (Herbst et al. 2014; Gerling et al. 2014). To date, we have not extended our work to include measures of CPTI activity or palmitate oxidation in either isolated mitochondria or muscle fibers from human skeletal muscle.

Mitochondrial content

Unlike previous reports in isolated skeletal muscle cells (Vaughan et al. 2012) and rodent skeletal muscle (Totland et al. 2000), omega-3 supplementation failed to induce mitochondrial biogenesis in human skeletal muscle. However, independent of changes in mitochondrial content, we recently reported that omega-3 supplementation can improve the ADP sensitivity of mitochondria in human skeletal muscle, representing a change in mitochondrial function (Herbst et al. 2014). Although the exact mechanisms are not apparent, we hypothesized that alterations in membrane composition or post-translational modifications in mitochondrial proteins may be responsible, indicating that omega-3s can affect physiological processes without altering mitochondrial content.

Placebo control

An area of constant debate surrounding studies examining the effect of omega-3 fatty acid supplementation is the use of a proper placebo (Golomb et al. 2010; Zulyniak et al. 2013). Generally, safflower oil or olive oil are given, both of which are high in unsaturated fatty acids. In the present study, the daily dosage was decreased to 3 capsules in the placebo group to limit any potential effects of olive oil (compared with 5 in the omega-3 group). The results from this study and similar studies in young (M.A. Zulyniak and D.M. Mutch 2014, personal communications) and older adults (Logan and Spriet 2014) demonstrated that this level of olive oil consumption did not affect any of the reported blood or whole-body measures.

Conclusion

This study demonstrated that supplementing with a high dose of omega-3s for 12 weeks increased RMR by 5.3% in a group of young healthy males. Individual responses in RMR were variable and appeared to be partially dependent on baseline RMR, and the data suggested that there are responders and nonresponders to omega-3 supplementation. In addition, when the data were normalized to body mass the significance was lost, suggesting that a small increase in FFM may have contributed to the increase in RMR.

Finally, omega-3 supplementation did not affect resting whole-body fat oxidation or increase the content of whole muscle, sarcolemmal, or mitochondrial fatty acid transport proteins or the mitochondrial content of human skeletal muscle.

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