Effects of eicosapentaenoic acid intake on denervation-induced mitochondrial adaptation in mouse skeletal muscle

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Abstract     Eicosapentaenoic acid (EPA) is an omega-3 polyunsaturated fatty acid that is abundant in fish oil, and has anti-inflammatory or anti-obesity effects. However, the effects of EPA supplementation on mitochondrial content and dynamics (fusion and fission) in skeletal muscle has not been elucidated. We investigated the effects of EPA intake for 4 weeks on denervation-induced mitochondrial adaptation in mice skeletal muscle. ICR mice (male, 8 weeks old) were daily administrated olive oil (control) or EPA at a dose of 300 mg/kg body weight by gavage for 4 weeks. After 2 weeks of oil intake, mice underwent unilateral sciatic nerve transection surgery. The hindlimb without surgery served as the sham-operated control. Body and skeletal muscle weights did not differ between the control and the EPA groups. Peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC-1α) and oxidative phosphorylation (OXPHOS) proteins were significantly decreased by denervation surgery. Denervation also induced the reduction of mitochondrial fusion molecules and the increase of mitochondrial fission molecules. Expression levels of PGC-1α and mitochondrial respiration protein MTCO1 were higher in the EPA group than in the control (olive oil) group. In addition, the EPA group contained higher levels of mitochondrial fusion protein OPA1. Our results suggest that EPA intake prevents the reduction of mitochondrial content and fusion proteins in denervated skeletal muscle.

Keywords: skeletal muscle, denervation, mitochondria, EPA

Introduction     Disuse of skeletal muscle causes the reduction of muscle mass and mitochondrial content¹. Sciatic denervation is a prevalent animal model utilized to induce muscle atrophy and mitochondrial degradation of the lower limbs². Furthermore, denervation induces the change of mitochondrial dynamics, accelerates mitochondrial fission and suppresses mitochondrial fusion³. Mitochondrial fission is regulated by fission protein 1 (FIS1) and dynamin protein 1 (DRP1), whereas mitochondrial fusion is regulated by mitofusin proteins (MFN1 and 2) and optic atrophy 1 (OPA1)⁴. DRP1 activity is modulated by the phosphorylation of serine 616⁵. Previous studies have demonstrated that disordered mitochondrial dynamics causes mitochondrial respiratory dysfunction⁶,⁷, suggesting that adequately balanced mitochondrial dynamics is important for maintaining mitochondrial function.

Eicosapentaenoic acid (EPA) is an omega-3 polyunsaturated fatty acid (PUFA) that is abundant in fish oil and has several effects, such as anti-inflammatory⁸ and anti-obesity⁹. Combined treatment of EPA and docosahexaenoic acid (DHA), which is an omega-3 PUFA also found in fish oil, enhanced the expression of the master regulator of mitochondrial biogenesis PGC-1α (peroxisome proliferator-activated receptor gamma coactivator 1 alpha) in non-muscle rhabdomyosarcoma (RMS) cells⁵, while in vivo work has reported that EPA increased mitochondrial size in rat skeletal muscle⁶. These studies provide some evidence that EPA may stimulate mitochondrial biogenesis; however, the effects of EPA intake on muscle inactivity-induced mitochondrial dysfunction has not been clearly elucidated. In this study, we focused on the effects of EPA intake on mitochondrial oxidative phosphorylation (OXPHOS) and mitochondrial dynamics regulatory proteins in denervated muscle.

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Materials and Methods

Animals and experimental design. All experimental procedures performed in this study were approved by the Institutional Animal Experiment Committee of the University of Tsukuba (approval number: 16-073). Eight-week-old male ICR (Institute of Cancer Research) mice (Tokyo Laboratory Animals Science Co., Japan) were used in this study. Animals were kept in temperature (22 ± 1°C)- and humidity (60 ± 10%)-controlled facilities under a 12 h (hour) light/dark cycle (07:00-19:00) and had ad libitum access to a normal diet (MF, ORIENTAL YEAST Co., Tokyo, Japan) and water. Mice were divided into control (olive oil; Wako, Osaka, Japan) or EPA (Maxomega™ EPA 97 EE, BASF, Land Rheinland-Pfalz, Germany) groups (n = 6 per group), and were given olive oil or EPA at a dose of 300 mg/kg body weight by gavage every day for four weeks. Olive oil has been used as a control or placebo in previous studies16,17). After two weeks of oil treatment, sciatic denervation surgery was undertaken as previously described18). Briefly, mice were anesthetized with isoflurane, and a small incision was made in the posterior aspect of the right hindlimb to expose the sciatic nerve at the level of the femoral trochanter. The exposed nerve portion (at least 5.0 mm) was excised and the skin closed with surgical glue. The left hindlimb then served as the sham-operated control. Following 2 weeks of denervation, mice were sacrificed by cervical dislocation, and the gastrocnemius, soleus, and extensor digitorum longus (EDL) muscles were dissected. The gastrocnemius muscles were minced by scalpel and equally separated in 1.5 mL tubes, frozen in liquid nitrogen, and stored at −80°C until analysis.

RNA isolation and quantitative real-time PCR. Approximately half of the gastrocnemius muscle sample was homogenized on ice in Trizol Reagent (Life Technologies, Gaithersburg, MD), and cDNA synthesis by PrimeScript™ RT (TAKARA, Shiga, Japan) was performed as per the manufacturer’s instructions. The expression of Pgc-1a (peroxisome proliferator-activated receptor gamma co-activator 1-alpha), Cox1 (cytochrome c oxidase subunit I), Cox4, Cyt c (Cytochrome c), Nd1 (NADH dehydrogenase subunit 1), Nd4, Mfn1 (mitofusin 1), Mfn2, Opa1 (optic atrophy 1), Fis1 (fission, mitochondrial 1), Drp1 (dynamin-related protein 1), were quantified using the Thermal Cycler Dice Real Time System and SYBR Premix Ex Taq II (Takara Bio, Shiga, Japan). Tbp (TATA box-binding protein) was used as a control housekeeping gene, the expression levels of which did not alter between the groups. Forward and reverse primers for the aforementioned genes are shown in Table 1.

Protein extraction and Western blotting. The remaining gastrocnemius muscle sample (approximately 50%) was homogenized in lysis buffer (50 mM HEPES (pH 7.4), 150 mM NaCl, 2 mM EDTA, 1% sodium deoxycholate, 1% NP-40, 0.2% SDS) with phosphatase inhibitor and a protease inhibitor mix (Nacalai Tesque Inc., Kyoto, Japan) on ice. Protein concentrations were measured using a Protein Assay Bicinchoninate Kit (Nacalai Tesque Inc.) and adjusted to 2.0 mg/mL with SDS-PAGE loading buffer (62.5 mmol/L Tris–HCl, pH 6.8, 2% w/v SDS, 10% glycerol, 50 mmol/L DTT, and 0.01% w/v bromophenol blue). An equivalent volume of each sample was loaded onto 10% polyacrylamide gel (PAGE). An electrically blotted PVDF membrane (Bio-Rad, Hercules, CA) was subjected

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer (5’-3’)</th>
<th>Reverse primer (5’-3’)</th>
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<tbody>
<tr>
<td>Tbp</td>
<td>CTGCCACACCAGCTTCTGGA</td>
<td>TGCAGAAATCGCTTGGG</td>
</tr>
<tr>
<td>Pgc-1a</td>
<td>TCCACCAAGAGGACAGTAT</td>
<td>CGCTGCCCATGAGGTATT</td>
</tr>
<tr>
<td>Cox1</td>
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<td>TGCCCAAGAACTGACAGC</td>
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<tr>
<td>Cox4</td>
<td>CTCAAAGCTTCTTTAGGAGC</td>
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</tr>
<tr>
<td>Cyt c</td>
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<td>CTCATTCCCTGCCC</td>
</tr>
<tr>
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<td>TGACGGACATCAAAAT</td>
</tr>
<tr>
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<td>ATTAGGATAGGCCA</td>
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<tr>
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<td>GCCTGTTCGAAGCAATAC</td>
<td>CACGGCCAGTGAAGACAT</td>
</tr>
<tr>
<td>Drp1</td>
<td>CGGTTCCTAAACTTACG</td>
<td>GCACATTTCCTTGTACG</td>
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to blocking with 1% bovine serum albumin in TBS containing 0.05% Tween 20 (TBST) for 1 h at room temperature (23 ± 2°C). Total OXPHOS Rodent WB Antibody Cocktail (ab110413), FIS1 (ab96764), DRP1 (ab56788), MFN2 (ab124773), 4-HNE (4-hydroxynonenal; ab48506) from Abcam (Cambridge, MA); Phospho-DRP1 (Ser616, #3455), OPA1 (#612606) from BD Transduction Laboratories (Tokyo, Japan); PGC-1α (No. 516557) from Millipore (Billerica, MA) were diluted 1:3000 with blocking buffer and used as the primary antibodies and incubated for 12 h at 4°C. Anti-mouse or anti-rabbit IgG-conjugated HRP (Life Technologies) was diluted 1:30000 with blocking buffer and used as the secondary antibody and incubated for 1 h at room temperature (23 ± 2°C). Bound antibody complexes were visualized using Immunostar LD (Wako) and C-Digit Blot Scanner (LI-COR, Lincoln, NE). Ponceau staining was used to verify consistent loading.

Enzyme activity. Maximal activity of citrate synthase (CS) in whole plantaris muscle was determined using the standard procedure described by Srere19).

Statistical analysis. All results were presented as mean ± standard error of means (SEM). Two-way analysis of variance (ANOVA) (denervation × oil) was performed, followed by Tukey’s post hoc test when an interaction was observed (GraphPad Prism for Mac OS X, Version 5.0f, La Jolla, CA). Statistical significance was defined as P < 0.05.

Results

There was no difference in body weight (immediately before sacrifice) between the control (olive oil) group (36.65 ± 0.95 g) and EPA group (36.45 ± 0.62 g). Denervation significantly decreased the wet weights of gastrocnemius, soleus, and EDL muscles in both the control and EPA groups; however, there was no difference between groups (Fig. 1). Denervation induced a significant reduction in mRNA levels of Pgc-1α and mitochondrial OXPHOS. mRNA expressions of Cox1 and Nd1 were significantly higher in the EPA group. Also, there was a trend in this direction in Pgc-1α (p = 0.06) and Cyt c (p = 0.10) (Fig. 2). At protein levels, PGC-1α and OXPHOS proteins (except UQCRC2) decreased by denervation. EPA ingestion showed significant higher expressions of PGC-1α and mitochondrial OXPHOS protein MTCO1, and SDHB (p = 0.07), UQCRC2 (p = 0.05) and ATP5A (p = 0.09) also approached significance (Fig. 2). Denervation resulted in decreased mRNA levels of mitochondrial fusion genes (Mfn1, Mfn2, and Opa1). Similarly, mitochondrial fusion protein MFN2 was decreased while fission protein FIS1 and both total and phosphorylated DRP1 were increased by denervation. The OPA1 protein level was significantly higher, and there was a trend towards an elevated Mfn1 mRNA level (p = 0.07) in the EPA group (Fig. 3). Maximal activity of CS was downregulated by denervation, however the effect of EPA supplementation was close to being significant (p = 0.05, Fig. 4). In order
to evaluate lipid peroxidation, we analyzed the level of 4-HNE content. Denervation led to robust lipid peroxidation, nevertheless there was no significant effect of EPA supplementation (Fig. 5).

Discussion

In the current study, we demonstrated that mitochondrial content indicator molecules were upregulated by EPA supplementation. Similarly, a previous study reported that EPA supplementation partially attenuated the age-related decline in mitochondrial function in mice\(^20\). We also found that EPA ingestion elevated mitochondrial fusion molecules OPA1 protein and \(\text{Mfn1}\) mRNA. In accordance with our observations, L6 myocytes cultured with DHA had a higher mitochondrial mass with a higher proportion of large and elongated mitochondria\(^21\). Moreover, high fish oil feeding increased OPA1 protein and induced the shift to elongated mitochondria observed in the electronic microscope analysis in rat liver\(^22\). These authors argued the possibility that fish oil/omega-3 PUFA might enhance mitochondrial fusion by receptor-mediated signaling and/or lipid composition. Additional research is needed to reveal detailed molecular mechanisms. Taken together, omega-3 PUFA supplementation appears to alter the mitochondrial dynamics toward fusion.

Interestingly, EPA supplementation improved endurance exercise capacity and skeletal muscle mitochondrial function in adult rats\(^23\). Although we did not measure the mitochondrial function, EPA supplementation may improve not only mitochondrial content, but also respiratory function possibly via altering protein dynamics. Mitochondrial fragmentation has also been shown to be associated with reactive oxygen species (ROS) formation\(^24\). Importantly, omega-3 supplementation increased the capacity for mitochondrial ROS emission by reorganizing the composition of mitochondrial membranes and promoting improvements in ADP sensitivity in human skeletal muscle\(^25\). The
Fig. 3 Effects of EPA ingestion on mitochondrial dynamics proteins in denervated muscle. 
(A-E) mRNA and (F-K) protein levels of mitochondrial fusion and fission regulatory proteins. Values represent the mean ± SEM (n = 6 per group). ** P < 0.01, significant effect of denervation. # P < 0.05, significant effect of EPA.

Fig. 4 Effects of EPA ingestion on maximal enzyme activity of CS. 
Values represent the mean ± SEM (n = 6 per group). ** P < 0.01, significant effect of denervation.

Fig. 5 Effects of EPA ingestion on 4-HNE content. 
(A) Typical western blotting band pattern and (B) protein levels of 4-HNE content. Values represent the mean ± SEM (n = 6 per group). ** P < 0.01, significant effect of denervation.
same group also reported that omega-3 supplementation increased uncoupling protein 3 in skeletal muscle.26)

While these results support the therapeutic potential of EPA supplementation, further studies will be required to further elucidate the underlying mechanisms of the effects of EPA and other omega-3 PUFAs.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this article.

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