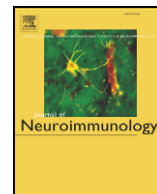




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Eicosapentaenoic acid decreases TNF- α and protects dystrophic muscles of *mdx* mice from degeneration

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ABSTRACT

In dystrophin-deficient fibers of *mdx* mice and in Duchenne muscular dystrophy, inflammation and increased production of tumor necrosis factor alpha (TNF- α) contribute to myonecrosis. We examined the effects of eicosapentaenoic acid (EPA) on dystrophic muscle degeneration. *Mdx* mice (14 days old) received EPA for 16 days. The sternomastoid, diaphragm and biceps brachii muscles were removed. Control *mdx* mice received vehicle. EPA decreased creatine kinase and myonecrosis and reduced the levels of TNF- α . These results suggest that EPA plays a protective role in dystrophic muscle degeneration, possibly by reducing TNF- α , and support further investigations of EPA as a potential therapy for dystrophinopathies.

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1. Introduction

The absence of dystrophin in Duchenne muscular dystrophy (DMD) and in the *mdx* mouse model of DMD is associated with sarcolemma instability and elevated levels of calcium in muscle fibers, factors that lead to myonecrosis. Muscle fiber damage activates an inflammatory response that seems to play a central role in the progression of myonecrosis (Tidball, 2005). Mast cells accumulate rapidly in response to muscle injury, followed by neutrophils and macrophages (Radley and Grounds, 2006). Progressive myonecrosis ultimately results in the replacement of muscle fibers with fat and fibrosis which, in turn, cause cardiorespiratory failure in dystrophic patients (Engel et al., 1994).

The proinflammatory cytokine tumor necrosis factor alpha (TNF- α), which is produced by inflammatory and muscle cells (Vassalli, 1992), is of particular importance for dystrophic fiber necrosis. TNF- α is found to be elevated in DMD and in *mdx* muscles (Kuru et al., 2003; Grounds et al., 2008) and higher serum levels are observed in DMD patients compared to healthy subjects (Saito et al., 2000). In addition, drug therapy designed to reduce inflammatory cells (Hodgetts et al., 2006), to block mast cell degranulation (Radley and Grounds, 2006) and to inhibit TNF- α and TNF-signaling mechanisms (Waters et al., 2010) has been shown to ameliorate dystrophy and demonstrates the role of inflammation and TNF- α in the progression of dystrophy.

The omega-3 fatty acid, eicosapentaenoic acid (EPA), has anti-inflammatory properties and several clinical trials have reported potential health benefits of omega-3 polyunsaturated fatty acids in many diseases, including cardiovascular diseases (Harper et al., 2006), epilepsy (Schlanger et al., 2002), inflammatory bowel disease (Calder, 2008), post-operative trauma (Roulet et al., 1997), exercise-trained subjects (Bloomer et al., 2009), and cancer-associated cachexia (Babcock et al., 2000). EPA has also been shown to inhibit the proinflammatory transcription factor nuclear factor kappa B (NF- κ B) (Babcock et al., 2000; Singer et al., 2008), to reduce TNF- α production by macrophages (Babcock et al., 2002) and to prevent the damaging effects of TNF- α during skeletal muscle differentiation *in vitro* (Magee et al., 2008).

There is currently no effective therapy for DMD. Corticosteroids are the main drugs of choice despite their side effects (Bonifati et al., 2000; Moxley et al., 2005) and long-term treatment is necessary. Despite advances in genetic and cell-based therapies (Tremblay et al., 2009; Heemskerk et al., 2010), the search for new drugs or nutritional interventions for DMD is relevant (Payne et al., 2006; Radley et al., 2007). In view of the anti-inflammatory and anti-TNF- α properties of EPA, we hypothesized that this agent would protect dystrophin-deficient *mdx* muscle fibers against degeneration.

2. Materials and methods

2.1. Animals

Male and female *mdx* mice (C57BL/10-*Dmd*^{*mdx*}/PasUnib) obtained from a breeding colony maintained by our institutional animal care

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facility were used in all experiments. Some C57BL/10 mice (C57BL/10ScCr/PasUnib) were used for focused experiments. The mice were housed according to institutional guidelines, with free access to food and water. Pregnant females were isolated and monitored daily. The date of birth was designated postnatal day 0. EPA treatment was initiated on postnatal day 14 before the cycles of muscle degeneration–regeneration had started (Cullen and Jaros, 1988). Mice were weaned at 4 weeks of age. The animal experiments described here were done in accordance with the guidelines of the Brazilian College for Animal Experimentation (COBEA; protocol # 2165-1) and the guidelines set forth by our Institution.

2.2. Drug administration

EPA-treated *mdx* mice ($n=15$) received EPA (cis-5,8,11,14,17-Eicosapentaenoic acid, 98.5% of EPA in composition, oil density 0.943 g/ml, Fluka/Sigma-Aldrich®, St. Louis, MO, USA) daily, by gavage, at a dose of 300 mg/kg body weight (Matsumoto et al., 2009) in 100% mineral oil (Nujol, liquid petrolatum for oral human use, Mantecorp, SP, Brazil) for 16 days. Each mouse was weighed daily so that the drug dosage could be adjusted accurately. The amount of oil received was 0.01 ml/gavage. Control litter *mdx* mice ($n=15$; untreated) received an equivalent amount of mineral oil.

2.3. Evans blue dye analysis

For visualization of muscle fiber leakiness/necrosis, treated and untreated-*mdx* mice were injected with Evans blue dye (EBD; Sigma®, St. Louis, MO, USA); (Matsuda et al., 1995). EBD was dissolved in phosphate-buffered saline (PBS; 0.15 M NaCl, 10 mM phosphate buffer, pH 7.4) and injected into the peritoneal cavity. The animals (5 EPA-treated and 5 untreated) received an intraperitoneal injection of 1% EBD in PBS at a dose of 100 μ l per 10 g of body weight. The mice were visually inspected for dye uptake. Discoloration of all mice was observed within 50–60 min after intraperitoneal injection of EBD, and successful injection of the dye was indicated by the blue color of the ears and paws.

Twenty-four hours later, the mice were anesthetized with a mixture of ketamine hydrochloride (130 mg/kg, Francotar, Virbac, São Paulo, Brazil) and xylazine hydrochloride (6.8 mg/kg, 2% Virbaxyl, Virbac, São Paulo, Brazil). The sternomastoid (STN), diaphragm (DIA), and biceps braquii (BB) muscles were dissected out and snap frozen in isopentane cooled in liquid nitrogen and stored at -80°C . These muscles were chosen because they are differently affected, with the diaphragm being more severely impaired than other muscles in the later stages of the disease (Stedman et al., 1991).

Cryostat cross-sections (7 μ m thick) were incubated in ice-cold acetone at -20°C for 10 min, washed three times for 10 min with PBS, and mounted in DABCO (mounting medium for fluorescence microscopy; Sigma). EBD staining shows a bright red emission upon fluorescence microscopy. Fiber counts of EBD-positive muscle fibers were performed with a hand counter in all sections and photographed under a Nikon fluorescence microscope connected to a Hamamatsu video camera. The number of EBD-positive muscle fibers is expressed as the percentage of the total number of muscle fibers counted in each section (4–5 sections for each muscle).

2.4. Quantitative and morphometric analysis

Cryostat cross-sections of STN, DIA and BB from EPA-treated ($n=7$) and untreated ($n=7$) were stained with hematoxylin–eosin (HE). Slides were placed in a Nikon Eclipse E 400 microscope connected to a personal computer and attached to a video camera (Nikon Express Series; Tokyo, Japan). Non-overlapping images of the entire cross-section were taken and tiled together using the ImagePro-Express software (Media Cybernetic; Silver Spring, MD).

For each cross-section (4–5 sections for each muscle), the numbers of central nucleated fibers and fibers with peripheral nuclei were counted using a hand counter and expressed as the percentage of the total number of fibers.

Areas with inflammatory cell infiltrate were characterized and quantified as previously described (Marques et al., 2008). In short, inflammatory cells were identified in hematoxylin–eosin sections based on nucleus morphology and cell size, showing basophilic nuclear staining and little cytoplasm. Areas containing inflammatory cells densely packed were measured with the ImagePro-Express software and were calculated as a percentage of the total muscle area in each section studied (4–5 sections from each muscle) using the microscope (Nikon Eclipse E400) fitted with a graduated eyepiece micrometer at 200 \times magnification. Some sections were labeled with antibody to F4/80 (Serotec; 1:250 dilution in 0.1 M PBS, pH 7.8, and BSA 1%), a pan macrophage marker (Villalta et al., 2009), followed by secondary antibody (CY-3; Jackson ImmunoResearch; 1:250 dilution in 0.1 M PBS, pH 7.8, and BSA 1%). Control mounts for the primary antibody were incubated with CY-3 anti-rat IgG in blocking solution instead of the primary antibody. No stained structures were seen in these controls. All the counting and measurements (EBD, central and peripheral nucleated cells and inflammation area) were done by a blinded observer.

2.5. Analysis of creatine kinase

For biochemical evaluation of muscle fiber damage, EPA-treated ($n=8$) and untreated ($n=8$) *mdx* mice were anesthetized with a mixture of ketamine hydrochloride (130 mg/kg, Francotar, Virbac, São Paulo, Brazil) and xylazine hydrochloride (6.8 mg/kg, 2% Virbaxyl, Virbac, São Paulo, Brazil). Blood samples (0.8 ml) were collected by cardiac puncture. After incubation at room temperature for 1–2 h to allow clotting, the samples were microcentrifuged at 936 g for 10 min, and the supernatant (serum) was removed and used for analysis. The creatine kinase (CK) assay was performed using a commercially available kit (CK Cinético Crystal, Bioclin, Quibasa, Minas Gerais, Brazil) and a Thermo Electron Corporation Genesys 20 spectrophotometer (Krackeler Scientific, Albany, New York, USA). Values are reported as international units (U/L).

2.6. Western blot analysis

TNF- α was quantified by western blotting in control C57BL/10 mice ($n=8$), and in EPA-treated ($n=8$) and untreated ($n=8$) *mdx* mice. The method employed was described in Ferretti et al. (2009). Briefly, muscles were lysed in assay lysis buffer (1% Triton, 10 mM sodium pyrophosphate, 100 mM NaF, 10 μ g/ml aprotinin, 1 mM PMSF, and 0.25 mM Na_3VO_4), centrifuged and the soluble fraction was resuspended in 50 μ l Laemmli loading buffer (2% SDS, 20% glycerol, 0.04 mg/ml bromophenol blue, 0.12 M Tris–HCl, pH 6.8, and 0.28 M β -mercaptoethanol). An amount of 30 μ g of aliquots from C57BL/10 and treated and untreated-*mdx* STN, DIA, and BB muscles was loaded onto 8%–15% SDS-polyacrylamide gels. Proteins were transferred from the gels to a nitrocellulose membrane using a submersion electrotransfer apparatus (Bio-Rad Laboratories, Hercules, California, USA). Membranes were incubated with the primary antibodies, followed by peroxidase-conjugated secondary antibodies and by development with SuperSignal West Pico Chemiluminescent Substrate kit (Pierce Biotechnology, Rockford, Illinois, USA). To control for protein loading, western blot transfer and nonspecific changes in protein levels, the blots were stripped and re-probed for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The luminescent signal from Western blot bands was captured by a G:Box iChemi camera (Syngene, Cambridge, UK) and band intensities were quantified using the analysis software provided by the manufacturer (Gene Tools Version 4.01, Syngene, Cambridge, UK).

The following primary antibodies were used for Western blotting: 1) TNF- α (rabbit anti-mouse polyclonal; Millipore, CA, USA), and 2) GAPDH (rabbit polyclonal; Santa Cruz Biotechnology, Santa Cruz, California, USA). The corresponding secondary antibody used for Western blotting was peroxidase-labeled affinity-purified rabbit IgG antibody (H + L) (KPL, Gaithersburg, Maryland, USA).

2.7. Statistical analysis

All data are expressed as means \pm standard deviation (SD). Statistical analysis for direct comparison between means of two groups was performed by the Student *t*-test and ANOVA was used for multiple statistical comparisons between groups. $P < 0.05$ was considered statistically significant.

3. Results

At the beginning of treatment (14 days of age), body mass was comparable among the groups studied (6.8 ± 0.5 g in untreated-*mdx* and 7.2 ± 0.7 g in EPA-*mdx*). There was a significant increase (about 45–50% compared to initial body mass) in body mass over time in both untreated- (10.0 ± 1.2 g; $p = 0.004$ compared to initial body mass, Student *t*-test) and EPA-treated (11.3 ± 2.0 g; $p = 0.02$ compared to initial body mass, Student *t*-test) *mdx*. Thus, EPA treatment did not interfere with the growth rate of young *mdx* mice.

3.1. Histopathology

The histological appearance of a muscle from normal C57BL/10 mice and from EPA-treated and untreated-*mdx* mice is shown in Fig. 1. In normal mice, muscle fibers with homogeneous diameter and peripheral nuclei located directly below the sarcolemma were observed. In the *mdx* mice, the inflammatory area was defined by areas containing conspicuous inflammatory cells densely packed among regenerated muscle fibers and muscle fibers under degeneration. Fully regenerated muscle fibers, characterized by central nucleation and an apparent normal diameter, predominated outside the inflammatory area. Groups of F4/80-expressing cells were present in all untreated-*mdx* muscles

(shown only in BB) and were decreased in the EPA-treated *mdx* mice (Fig. 1).

3.2. Analysis of myonecrosis: Evans blue dye, central nucleated fibers and CK

EPA caused a significant decrease in Evans blue dye staining, indicative of sarcolemma leakiness and myonecrosis, with a concomitant increase in fibers with peripheral nuclei, in all muscles studied (Table 1). There was a reduction of central nucleated fibers in the EPA-treated muscles, with a significant decrease seen only in STN muscle (Table 1), possibly due to the higher levels of these fibers in the untreated-STN in comparison to BB and DIA. CK levels were significantly decreased by EPA compared to untreated-*mdx* mice (838 ± 347 U/L in EPA-treated vs 1208 ± 376 U/L in untreated-*mdx*; $p = 0.04$, Student's *t*-test).

3.3. Analysis of inflammation: inflammatory area and TNF- α levels

Quantitative data about the inflammatory area is shown in Fig. 2. While untreated DIA presented the lowest inflammatory area (13%), limb and STN muscles showed the highest values, with about 65% (BB) to 100% (STN) increase in comparison to DIA. EPA decreased the inflammatory area in all muscles studied (Fig. 2).

The levels of TNF- α in dystrophic STN, DIA and BB were significantly higher in untreated-*mdx* muscles in comparison to normal C57BL/10 mice (Fig. 3). The highest level of TNF- α was observed in STN, followed by BB and DIA (Fig. 3). EPA led to a significant reduction in the levels of TNF- α in all muscles (about 50% reduction in STN and BB and 25% in DIA; Fig. 3).

4. Discussion

EPA is a 20-carbon omega (n)-3 polyunsaturated fatty acid with anti-inflammatory properties, which is synthesized from ingested alpha-linolenic acid or is consumed in fish and fish oil (Arterburn et al., 2006). The present study demonstrated that treatment of *mdx* mice with EPA resulted in a decrease of serum CK levels and Evans blue dye-positive fibers and a concomitant increase in peripheral

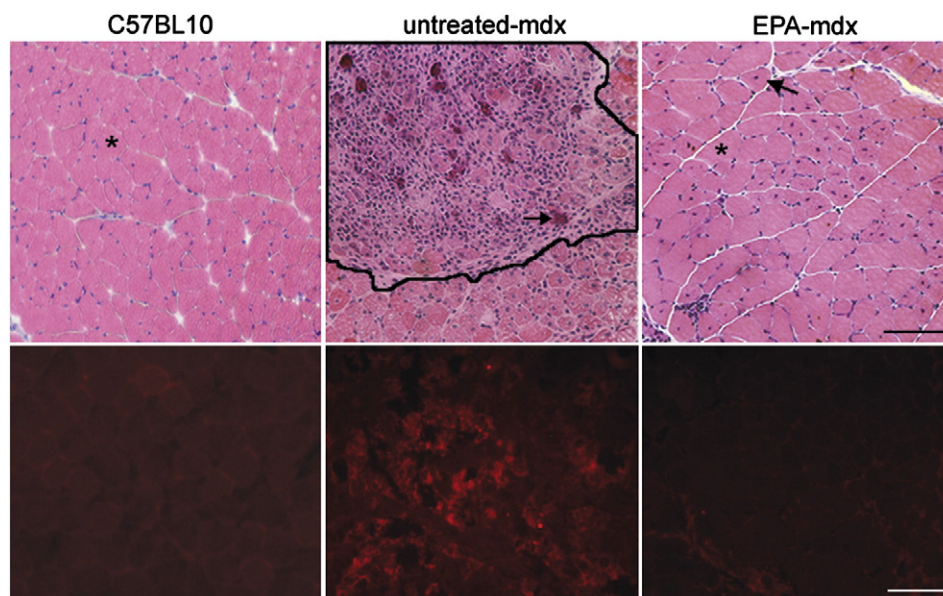


Fig. 1. Histological appearance of control C57BL/10, untreated and EPA-treated *mdx* mice. Upper row: sternomastoid muscle stained with hematoxylin–eosin. Fiber with peripheral cell nuclei (asterisk) in control and in EPA-treated muscles. In untreated-*mdx*, a representative inflammatory area is indicated by the outline, with necrotic fibers (arrow). In EPA-*mdx*, central nucleated fiber (arrow). Bottom row: representative areas of biceps braquii muscle labeled with F4/80 antibody. Groups of F4/80-expressing cells are seen in untreated-*mdx* mice. Scale bar: 60 μ m.

Table 1
Effects of EPA treatment on the percentage of muscle fibers with peripheral cell nuclei (peripheral nuclei), centrally located nuclei (central nuclei), and fibers stained with Evans blue dye (EBD).

		Total number of fibers	Peripheral nuclei (%)	Central nuclei (%)	EBD (%)
STN	Untreated	966 ± 52.27	50.0 ± 4.5	34.3 ± 2.3	15.6 ± 2.6
	Treated	882 ± 84.22	70.5 ± 5.5* p<0.001	25.2 ± 5.3* p<0.001	4.2 ± 2.2* p<0.001
DIA	Untreated	1250 ± 90.5	73.4 ± 6.6	10.2 ± 3.5	16.3 ± 7.0
	Treated	1320 ± 94.2	86.7 ± 4.3* p<0.001	8.0 ± 2.7	5.2 ± 2.7* p=0.004
BB	Untreated	961 ± 88.6	71.7 ± 8.3	18.6 ± 7.3	9.3 ± 3.6
	Treated	1183 ± 109.35	83.3 ± 3.0* p=0.03	13.8 ± 3.0	4.2 ± 1.8* p=0.01

Values are expressed as the percentage (mean ± SD; n = 5 mice) of the total number of fibers of the sternomastoid (STN), diaphragm (DIA) and biceps brachii (BB) muscles.

* Significantly different from untreated-*mdx* mice within the same muscle group (Student *t*-test).

nucleated fibers, evidence indicating a decline in myonecrosis. Taken together, these results show that EPA treatment exerts a protective effect on myofiber breakdown in limb (BB) and axial (STN and DIA) muscles of young *mdx* mice.

In the present study, the dose of EPA used (300 mg/kg) seems to be higher than might be expected to human patients, where a 1000 mg/day of EPA is usually recommended. However, care must be taken to extrapolate dose from mice to humans, especially if we consider differences in metabolism, in the duration of treatment and in the progression of dystrophy. In an experimental cachexia model in rats, it was observed that EPA inhibited tumor growth and body weight loss in a dose-dependent manner, with optimal effects seen at higher doses (1.25–2.5 g/kg body weight) than that used here. Toxicity was observed at a dose of 5 g/kg (Tisdale and Dhesi, 1990). In patients with advanced pancreatic cancer, the oral intake of a nutritional supplement enriched with 1.09 g of EPA, twice a day, attenuated the progression of cachexia (Barber et al., 1999). Thus, considering that DMD patients may need a long therapy, the lower recommended dose should be more appropriate, but clinical experiments are necessary to properly address this question.

Inflammation is a hallmark of dystrophic muscles and strongly contributes to the progression of the disease (Chen et al., 2000; Porter

et al., 2002). Although COX-2-derived eicosanoids do not seem to contribute to the progression of dystrophic disease (Pierno et al., 2007), the inhibitory action of EPA on arachidonic acid metabolism through the 5-lipoxygenase and cyclooxygenase enzymatic pathways, suppressing the production of n-6 eicosanoid inflammatory mediators (James et al., 2000; Calder, 2008), may explain at least in part the reduced muscle damage seen in dystrophic *mdx* muscle fibers.

In dystrophic muscles, mast cells rapidly accumulate once muscle damage begins and macrophages are predominant between 12 h to 24 h after the injury (Radley and Grounds, 2006). Distinct macrophage populations may influence the course of *mdx* dystrophy (Villalta et al., 2009) and both, mast cells and macrophages, produce proinflammatory cytokines such as TNF- α . We observed F4/80-expressing cells in all dystrophic muscles studied, at this early stage of dystrophy, further supporting the primary roles of macrophages in *mdx* dystrophy (Villalta et al., 2009; Wehling et al., 2001). It is well known that inhibition of TNF- α production protects dystrophin-deficient *mdx* fibers against muscle degeneration (Radley and Grounds, 2006; Waters et al., 2010). In the present study, EPA reduced immunofluorescence labeling of macrophages, reduced significantly TNF- α levels in all muscles studied and ameliorated myonecrosis, findings indicating a role of macrophages and TNF- α in the early stages of disease in muscles that are affected differently by the lack of dystrophin. The present findings are in agreement with previous studies showing that EPA is able to decrease TNF- α levels by inhibiting its production by macrophages (Babcock et al., 2002), and also to reduce serum TNF- α levels in exercised humans (Bloomer et al., 2009).

The mechanisms whereby EPA exerts its anti-TNF effects are poorly understood. One possibility is that EPA decreases transcription of the TNF- α gene by reducing the translocation of NF- κ B, a proinflammatory transcription factor (Jousen et al., 2002), from the cytoplasm to the nucleus (Lo et al., 1999; Singer et al., 2008). Disturbances in NF- κ B signaling have been described in *mdx* mice (Acharyya et al., 2007) and in DMD (Monici et al., 2003). EPA may prevent the damaging effects of TNF- α on muscle fibers as demonstrated in *in vitro* studies on skeletal muscle differentiation (Magee et al., 2008). Additionally, EPA may improve dystrophic muscle regeneration, since it was demonstrated that this omega-3 fatty acid regulates myogenic regulatory factors and attenuates muscle wasting by preventing muscle protein degradation (Castillero et al., 2009).

In conclusion, the present study demonstrated that the administration of highly purified EPA to dystrophic *mdx* mice decreases inflammation, myonecrosis and TNF- α levels in different dystrophin-deficient skeletal muscles. EPA has been tested in several clinical trials for different diseases and its effects in reducing the risk of cardiac death are well documented (Nestel, 2000; Harper et al., 2006). Although further studies are necessary to better understand the mechanisms whereby EPA modulates immunoinflammatory pathways in dystrophic muscle, the present results suggest that therapeutic strategies using EPA as a supplemental or primary therapy may ameliorate dystrophic muscle pathology.

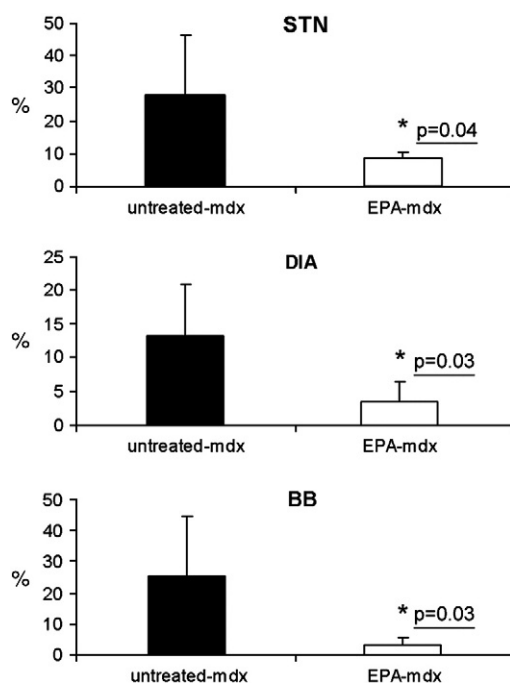


Fig. 2. Quantitation of inflammatory area in sternomastoid (STN), diaphragm (DIA) and biceps brachii (BB) muscles from untreated (n = 7) and EPA-treated (n = 7) *mdx* mice. The inflammatory area is represented as a percentage of the total muscle area. *Means significantly different from untreated-*mdx*. Student *t*-test. Error bars, SD.

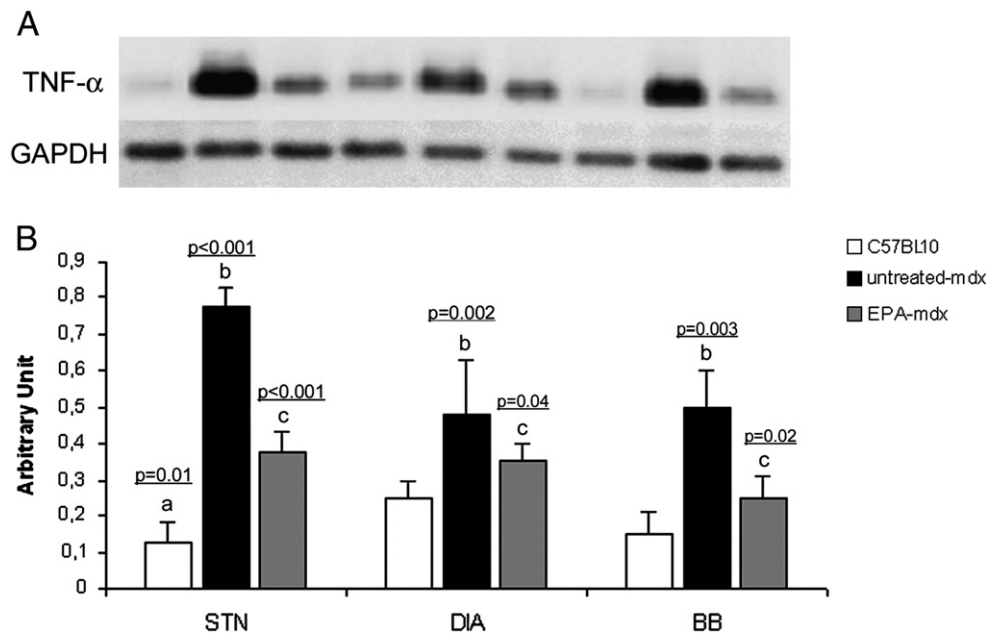


Fig. 3. Immunoblot analysis of tumor necrosis factor (TNF)-alpha levels in crude extracts of sternomastoid (STN), diaphragm (DIA), and biceps brachii (BB) muscles from control C57BL/10 ($n=8$), and from untreated ($n=8$) and EPA-treated ($n=8$) *mdx* mice. A. Western blot. B. Quantitation shows higher levels of TNF- α in dystrophic muscles compared to normal mice (untreated \times C57BL/10). EPA led to a significant reduction in TNF- α levels in all muscles. Levels of TNF- α expressed in arbitrary units normalized to the levels of GAPDH, which was used as a loading control. Significantly different from EPA-*mdx* (a), from C57BL/10 (b) and from untreated-*mdx* (c), ANOVA. Error bars, SD.

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