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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.

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-kB) (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 μl 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 μm 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 hematoxilin–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 hematoxilin–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× 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 μg/ml aprotinin, 1 mM PMSF, and 0.25 mM Na_3VO_4), centrifuged and the soluble fraction was resuspended in 50 μl 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 ß-mercaptoethanol). An amount of 30 μg 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).