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EPA protects against muscle damage in the *mdx* mouse model of Duchenne muscular dystrophy by promoting a shift from the M1 to M2 macrophage phenotype

Samara Camaçari de Carvalho [a], Leticia Montanholi Apolinário [a], Selma Maria Michelin Matheus [b], Humberto Santo Neto [a], Maria Julia Marques [a,*]

[a] Departamento de Biologia Estrutural e Funcional, Instituto de Biologia, Universidade Estadual de Campinas (UNICAMP), Campinas, São Paulo 13083-970, Brazil
[b] Departamento de Anatomia, Instituto de Biociências, Universidade Estadual Paulista Julio de Mesquita Filho, Botucatu, São Paulo 18618000, Brazil

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ABSTRACT

In dystrophic *mdx* mice and in Duchenne muscular dystrophy, inflammation contributes to myonecrosis. Previously, we demonstrated that eicosapentaenoic acid (EPA) decreased inflammation and necrosis in dystrophic muscle. In the present study, we examined the effects of EPA and the corticoid deflazacort (DFZ) as modulators of M1 (iNOS-expressing cells) and M2 (CD206-expressing cells) macrophages. *Mdx* mice (14 days old) received EPA or DFZ for 16 days. The diaphragm, biceps brachii and quadriceps muscles were studied. Immunofluorescence, immunoblotting and ELISA assays showed that EPA increased interleucin-10, reduced interferon-γ and was more effective than DFZ in promoting a shift from M1 to M2.

1. Introduction

Duchenne muscular dystrophy (DMD) is the most common lethal genetic disease of childhood that results from the lack of dystrophin, a sarcolemmal protein that binds the cytoskeleton of skeletal muscle to the extracellular compartment (Hoffman et al., 1987; Engel et al., 1994). In DMD and in the *mdx* mouse model of DMD, loss of dystrophin causes sarcolemmal instability and an increased influx of calcium (Whitehead et al., 2006), resulting in progressive myonecrosis followed by fibrosis. Fibrosis causes cardiorespiratory failure and ultimately results in the death of DMD patients in approximately their third decade of life (Engel et al., 1994). The progressive muscle injury that occurs in dystrophic muscles is exacerbated by the inflammatory response of a heterogeneous infiltrate of immune cells that produces and releases pro-inflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α), interleukins (ILs) and interferon-γ (IFN-γ) (Porter et al., 2002; Tidball, 2005; Hodgetts et al., 2006; Villalta et al., 2011a).

The inflammatory infiltrate in dystrophic skeletal muscles is composed of macrophages, T-cells, neutrophils, eosinophils and mast cells. Macrophages are the major leukocyte present between 24 and 48 h after injury (Gorospe et al., 1996; Radley and Grounds, 2006), and they play a central role in the pathogenesis of muscular dystrophy. Two distinct populations of macrophages have been described in dystrophic muscles, M1 and M2 types (Villalta et al., 2009). M1 macrophages participate in Th1 immune responses and are able to damage dystrophic muscles; M2 macrophages participate in Th2 immune responses and can promote muscle regeneration (Mantovani et al., 2004; Villalta et al., 2009). Therefore, drugs that can deactivate the M1 phenotype and promote activation of the M2 phenotype can increase muscle repair and ameliorate the progression of muscular dystrophies (Kovalovskya et al., 2000; Mikita et al., 2011; Villalta et al., 2011b; Deng et al., 2012).

The anti-inflammatory corticosteroids deflazacort and prednisone are the therapies of choice for DMD given their ability to protect against muscle damage and slow down the progression of the disease (Manzur et al., 2008; Bushby et al., 2010). However, due to significant side effects associated with deflazacort and prednisone treatments, the search for alternative drugs is important. Omega-3 and other nutritional compounds that show anti-inflammatory properties have been used as a drug therapy or nutritional intervention to ameliorate several inflammatory pathologies (Gil, 2002; Payne et al., 2006; Radley et al., 2007; Matsumoto et al., 2009; Hao et al., 2010; Calder, 2012). Previously, we demonstrated that eicosapentaenoic acid (EPA) ameliorated dystrophinopathy in *mdx* mice by decreasing skeletal muscle necrosis and inflammation, with a concomitant decrease in the levels of TNF-α (Machado et al., 2011).

In the present study, we hypothesized that the protective effects of EPA on dystrophic muscle may be related, at least in part, to their ability

* Corresponding author. Tel.: +55 19 3521 6395; fax: +55 19 3521 6185.
E-mail address: marques@unicamp.br (M.J. Marques).

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to increase the macrophages with the M2 regenerative phenotype and to decrease the M1 cytolytic phenotype. We were also interested in comparing the effects of EPA to the effects of deflazacort on the M1 and M2 macrophage populations in *mdx* mice. Our aim was to provide further support for the use of EPA as a potential treatment of dystrophinopathies that merits testing in DMD clinical trials.

2. Materials and methods

2.1. Animals

Male and female *mdx* mice (C57BL/10-*Dmd*^{mdx}/PasUnib) and control C57BL/10 mice (C57BL/10ScCr/PasUnib) were used in all experiments. They were obtained from a breeding colony maintained by the Multidisciplinary Center for Biological Research (CEMIB, University of Campinas). 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 conducted in accordance with the guidelines of the Brazilian College for Animal Experimentation (COBEA; protocol # 2232-1) and the guidelines set forth by our institution.

2.2. Drug administration

EPA-treated *mdx* mice (n = 18) received cis-5,8,11,14,17-eicosapentaenoic acid (EPA, purity 98.5%; density 0.943 g/ml; Fluka/Sigma-Aldrich®, St. Louis, MO, USA) daily by gavage at a dose of 300 mg/kg body weight in 100% mineral oil (Nujol, liquid petrolatum for oral human use, Mantecorp, SP, Brazil) for 16 days, as previously described (Matsumoto et al., 2009; Machado et al., 2011). Each mouse was weighed daily, and drug dosage was adjusted accordingly. The volume of oil received was 0.01 ml/gavage. Deflazacort (DFZ)-treated *mdx* mice (n = 18 mice) received DFZ (Libbs, Brazil) daily by gavage at a dose of 1.2 mg/kg in water for 16 days, as previously described (Keeling et al., 2007; Marques et al., 2009). Control litter *mdx* mice (n = 18; untreated) received an equivalent amount of mineral oil.

2.3. Morphometric analysis

Cryostat cross-sections of diaphragm (DIA), biceps braquii (BB) and quadriceps (QDR) from untreated *mdx* mice (n = 10) were used to characterize the overall histopathology (HE staining) and the M1 and M2 composition (immunofluorescence staining) of the inflammatory infiltrate. These muscles were chosen because limb (BB and QDR) and axial (DIA) muscles, which they represent, are affected differently by the lack of dystrophin, with the DIA being the most affected in the course of the disease (Stedman et al., 1991). Slides were examined under a Nikon Eclipse E 400 microscope (Nikon Express Series; Tokyo, Japan) fitted with a graduated eyepiece micrometer at 200× magnification. Non-overlapping images of the entire cross-section were taken and tiled together using ImagePro-Express software (Media Cybernetic; Silver Spring, MD).

The areas with inflammatory cell infiltrate were characterized by immunofluorescence. As previously described (Villalta et al., 2009), inflammatory areas were labeled with F4/80 (Serotec; 1:250 dilution in 0.1 M PBS, pH 7.8 and BSA 3%), a pan macrophage marker, followed by secondary antibody (Texas Red, Vector Laboratories, Burlingame, California, USA; 1:200 dilution in 0.1 M PBS, pH 7.8, and BSA 1%). The M1 macrophage phenotype was labeled with iNOS (Sigma-Aldrich, Saint Louis, Missouri, USA; 1:250 dilution in 0.1 M PBS, pH 7.8, and BSA 3%) followed by secondary antibody (Anti-mouse FITC, Sigma-Aldrich, Saint Louis, Missouri, USA; 1:200 dilution in 0.1 M PBS, pH 7.8, and BSA 1%) and the M2 macrophage phenotype was labeled with CD206 antibody (AbD Serotec, Kidlington, Oxford, UK; 1:250 dilution in 0.1 M PBS, pH 7.8, and BSA 3%) followed by fluorescein conjugated anti-rat antibody (Vector Laboratories, Burlingame, California, USA; 1:200 dilution in 0.1 M PBS, pH 7.8, and BSA 1%). Adjacent cross-sections were labeled with antibodies against F4/80, iNOS and CD206 to characterize macrophages with M1 and M2 phenotypes.

The inflammatory areas from untreated-, EPA- and DFZ-treated *mdx* mice were measured using ImagePro-Express software and expressed as a percentage of the total muscle area in each section studied (2 sections from each muscle), as previously described (Machado et al., 2011). Given that the two types of macrophages were mainly distributed into clusters (Fig. 2), the proportions of the two cell types were also quantified by area measurements. Areas containing M1 or M2 macrophages from untreated-, EPA- and DFZ-treated *mdx* mice were measured as described and expressed as a percentage of the total inflammatory area in each section studied. Control mounts for the primary antibodies were incubated with respective secondary antibodies in blocking solution instead of the primary antibody. No stained structures were observed in these controls. The same observer, blinded with respect to treatment, performed all the measurements (total inflammatory area and M1 and M2 areas).

2.4. Analysis of creatine kinase in serum

An assay for creatine kinase (CK) was performed as previously described (Machado et al., 2011). In short, EPA-treated (n = 10), DFZ-treated (n = 10) and untreated (n = 10) *mdx* mice were anesthetized using 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, the samples were microcentrifuged at 936 *g* for 10 min and the supernatant (serum) was removed and used for the biochemical analysis of muscle degeneration. The 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).

2.5. Western blot analysis

iNOS (M1), CD206 (M2) and F4/80 (total inflammatory area) were quantified by Western blotting in control C57BL/10 mice (n = 8) and EPA-treated (n = 8), DFZ-treated (n = 8) and untreated (n = 8) *mdx* mice. The method employed was described in Machado et al. (2011). Briefly, muscles were lysed in assay buffer (1% Triton, 10 mM sodium pyrophosphate, 100 mM NaF, 10 μg/ml aprotinin, 1 mM PMSF, and 0.25 mM Na_3VO_4) and centrifuged. 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). Aliquots (30 μg) from the DIA, BB and QDR muscle fractions of C57BL/10 mice and treated and untreated *mdx* mice were 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 then they were developed using 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 the 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).