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Nicotinic acetylcholine receptor activation reduces skeletal muscle inflammation of *mdx* mice

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ABSTRACT

Mdx mice develop an inflammatory myopathy characterized at different ages by myonecrosis with scattered inflammatory infiltrates followed by muscular regeneration and later persistent fibrosis. This work aimed to verify the putative anti-inflammatory role of nicotinic acetylcholine receptor (nAChR) in the *mdx* muscular lesion. Mitigation of myonecrosis and decreased TNFα production were accompanied by increased numbers of F4/80 macrophages expressing nAChRα7. In vivo treatment with nicotine attenuated muscular inflammation characterized by reduced metalloprotease MMP-9 activity, TNFα and NFkB content and increased muscular regeneration. Our data indicate that nAChR activation influences local inflammatory responses in the muscular lesion of *mdx* mice.

1. Introduction

Duchenne muscular dystrophy (DMD) is a devastating X-linked recessive inflammatory myopathy in which progressive muscle degeneration is caused by a defect in the gene coding for dystrophin, a large cytoskeletal protein present in skeletal muscles and certain neurons (Voisin and de la Porte, 2004). At the sarcolemma, the dystrophin glycoprotein complex (DGC) shares a receptor for the extracellular matrix components laminin and agrin providing an important framework for spindle fiber differentiation, clustering and consolidation of acetylcholine receptors (AChRs) at the neuromuscular junction (Carlson, 1998; Williams and Jacobson, 2010). Lack of dystrophin causes sarcolemmal instability predisposing to myonecrosis and activation of inflammatory signalling cascades. Although inflammation is the pathological hallmark of dystrophic muscular lesion, the mechanisms influencing muscle fiber pathology are still not understood (Evans et al., 2009). Dystrophinopathy in *mdx* mice is characterized by progressive muscle wasting, high plasma concentration of creatine kinase due to substantial muscle necrosis, calcium-dependent endplate degeneration and atrophy (Torres and Duchen, 1987). Cycles of myonecrosis with scattered inflammatory infiltrates and muscular regeneration are evident between 3 and 4 weeks followed by persistent fibrosis and accumulation of connective tissues from 12 weeks onwards in older *mdx* mice (Lagrota-Candido et al., 2002; Lefaucher and Sebille, 1996; McGeachie and Grounds, 1999).

Inflammatory cells present a complete cholinergic system consisting of acetylcholine (ACh), muscarinic and nicotinic receptors, choline acetyl-transferase and acetyl-cholinesterase (Kawashima and Fujii, 2003; Tayebati et al., 2002). Nicotine acetylcholine receptors (nAChRs) belong to the family of nicotinic acetylcholine ligand-gated ion channels and have five subunits forming hetero or homopentameric receptors with different pharmacological properties (Gotti and Clementi, 2004). Cholinergic stimulation of the α7 homopentameric nAChR inhibits production of pro-inflammatory cytokines (Bernik et al., 2002; Borovikova et al., 2000; Nizri et al., 2006; Saeed et al., 2005; Wang et al., 2003), expression of endothelial cell adhesion molecules, and leukocyte recruitment during inflammation (Saeed et al., 2005). The anti-inflammatory effect of nAChRα7 stimulation is partly related to the regulation of cytokine production by macrophages (Bernik et al., 2002; Borovikova et al., 2000; Rosas-Ballina et al., 2009; Saeed et al., 2005; Shytle et al., 2004; Ulloa, 2005; Wang et al., 2003) and T lymphocytes (Nizri et al., 2006).

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Presence of acetylcholine as the neurotransmitter of cholinergic system at sites of nerve–muscle contacts establishes a link between immune and nervous system which might be influencing the pathogenesis of *mdx* muscular lesion. In the present study, we correlated nAChRα7 expression with presence of F4/80 macrophages and local production of the inflammatory cytokine tumor necrosis factor alpha (TNFα) at different stages of the disease and further determined the effect of in vivo stimulation of nicotinic acetylcholine receptors upon inflammation and muscular regeneration of *mdx* mice.

2. Materials and methods

2.1. Animal care

Male *mdx* dystrophic and age-matched C57BL/10 J control non-dystrophic mice were maintained at the animal house facilities of the Department of Cellular and Molecular Biology at Fluminense Federal University. Mice were kept at constant temperature (20 °C) with a light/dark cycle of 12 h. Each cage housed up to 4 mice of the same age, gender and offspring to minimize stress. Mice were sacrificed at ages 2, 4, 12 and 24 weeks. All procedures were approved by the Institutional Animal Care (protocol 174-09) and were conducted according to Brazilian Ethics Guidelines for Animal Studies (COBEA).

2.2. Histological staining and morphometric analysis

Gastrocnemius muscles from *mdx* and control mice were carefully removed, fixed in formalin-buffered Millonig fixative (pH 7.2) for 24 h. Wax-embedded 5 μm-thick sections were stained with hematoxilin–eosin and sirius red for collagen. Images of all cross-sections from five *mdx* and control mice at each time point were acquired with a microdigital camera mounted on a Zeiss Axioplan microscope (Zeiss, Oberkochen, Germany) using a 20× objective. Images were mounted with Photomerge Adobe Photoshop CS3 software. Total surface area and areas occupied by inflammatory infiltrates, regeneration and collagen deposition were determined with Image-Pro 4.5 software. Regenerating fibers were identified by strong basophilic sarcoplasma and centrally located nuclei. Results were expressed as percentage of total area in the cross-section.

2.3. Immunohistochemistry

Mice were deeply anesthetized by intraperitoneal injection of (1:1) ketamine hydrochloride (Ketalar; Parke-Davis, São Paulo, Brazil) and xylazine hydrochloride (Rompun; Bayer, São Paulo, Brazil), perfused with 20 mL 0.25% heparin (Actparin; Bergamo, São Paulo, Brazil) in saline through the ascending aorta followed by 30 mL cold buffer fixative (4% paraformaldehyde in 100 mM PBS, pH 7.4). Muscles were carefully removed, placed for 4 h in 4% paraformaldehyde solution and submitted to cryoprotection by sequential incubation with a gradient of sucrose solution (10%, 20% and 30% w/v in PBS) for 6 h at 4 °C in each solution. Cryostat cross-sections (10 μm, spaced 200 μm) were mounted on poly-L-lysine pre-coated slides, rinsed for 20 min in PBS and incubated for 2 h with blocking buffer PBST (0.05% Triton X-100 in PBS, containing 5% normal goat or horse). For nAChRα7 detection, sections were incubated overnight at 4 °C with primary monoclonal rat anti-nAChRα7 (clone 319; Covance, Princeton, NJ) at 1:250 dilution in PBS followed by incubation with goat anti-rat IgG FITC (1:300 dilution; Invitrogen, Carlsbad, CA) for 2 h at 37 °C. Sections were further incubated during 2 h with rat anti-F4/80 biotinylated primary antibody at 1:40 dilution (clone A3-1 C57BL; Serotec, Oxford, UK) for detection of macrophages (Khazen et al., 2005), followed by 1 h incubation with streptavidin Cy3–conjugate (1:1000 dilution; Jackson Immuno Research; West Grove, PA) at room temperature. Muscular regeneration was evidenced by overnight incubation at 4 °C with goat anti-NCAM antibody (1:200 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) followed by incubation for 1 h at 37 °C with donkey anti-goat IgG Alexa 488-labeled antibody (1:200 dilution; Molecular probes, Eugene, OR). TOPRO-3 (Molecular probes, Eugene, OR) was always used to counterstain cellular nuclei. Images randomly collected from seven inflammatory foci or regeneration areas at each time point were obtained with Zeiss LSM510 META confocal microscope with identical time exposure and image settings. Analysis was performed using image-analysis software AxioVert 40 (Zeiss, Oberkochen, Germany). Five animals were included at each time point.

2.4. Western blotting

Skeletal muscles were homogenized with protease inhibitor buffer (Sigma, St Louis, MI). Protein extracts were clarified by centrifugation at 12,000×*g* for 15 min at 4 °C, and quantification was determined by the Lowry method (Lowry et al., 1951) followed by concentration adjustment with sample buffer, pH 6.8 (173 mM Tris, 30% glycerol, 3% sodium dodecyl sulfate, 3% β-mercaptoethanol, and 0.1% bromophenol blue). Samples were denatured by boiling for 5 min and loaded on 10% SDS-PAGE for nAChRα7 and myogenin, on 12.5% for TNFα and 7.5% for NCAM detection. Proteins were transferred to PVDF membranes (Hybond-P; Amersham Biosciences, Fairfield, CT) and blocked for 2 h at room temperature with 5% nonfat dry milk in 0.05% Tween 20 Tris–buffered saline (TBST), pH 7.4 on a rocking platform. Membranes were incubated with specific rat monoclonal anti-nAChRα7 (clone 319 at 1:400 dilution; Covance, Princeton, NJ); anti-TNF biotinylated antibodies (clone MP6-XT3 at 1:3000 dilution; BD-Pharmingen Biosciences, San Diego, CA); polyclonal rabbit anti-NFkB p65 (at 1:500 dilution; Santa Cruz Biotechnology, Santa Cruz, CA); anti-myogenin (at 1:1000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA); and polyclonal goat anti-human NCAM (at 1:400 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) in 5% nonfat dry milk on TBST at 4 °C overnight. After washes with TBST, blots were incubated for 2 h with goat anti-rat peroxidase conjugated (Sigma, St Louis, MI) at 1:5000 dilution for nAChRα7, avidin-peroxidase conjugated (BD-Pharmingen Biosciences, San Diego, CA) at 1:3000 dilution for TNFα, goat anti-rabbit peroxidase conjugated (Southern Biotechnology Associates Inc., Birmingham, AL) at 1:3000

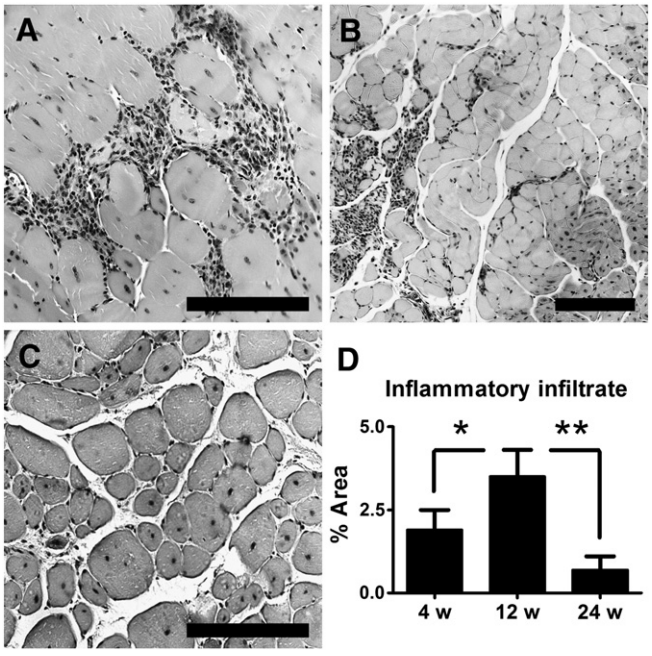

Fig. 1. Morphometric analysis of inflammatory infiltrates in *mdx* skeletal muscle. Histological analysis of *mdx* gastrocnemius muscles at (A) 4 weeks, (B) 12 weeks and (C) 24 weeks. Scale bar 100 μm. (D) Percentage of inflammatory infiltrate area with statistical unpaired *t*-test analysis (* $p<0.05$; ** $p<0.01$). Results from 5 animals per group are expressed as mean with standard deviation (±SD).