Neurobiology of Disease 32 (2008) 243–253

Contents lists available at ScienceDirect

Neurobiology of Disease

journal homepage: www.elsevier.com/locate/ynbdi

Gentamicin treatment in exercised mdx mice: Identification of dystrophin-sensitive pathways and evaluation of efficacy in work-loaded dystrophic muscle

Annamaria De Luca [a,*], Beatrice Nico [b], Jean-François Rolland [a], Anna Cozzoli [a], Rosa Burdi [a], Domenica Mangieri [b], Viviana Giannuzzi [a], Antonella Liantonio [a], Valentina Cippone [a], Michela De Bellis [a], Grazia Paola Nicchia [c], Giulia Maria Camerino [c], Antonio Frigeri [c], Maria Svelto [c], Diana Conte Camerino [a]

[a] Unit of Pharmacology, Department of Pharmacobiology, Faculty of Pharmacy, University of Bari, Italy
[b] Department of Human Anatomy and Histology, University of Bari, Italy
[c] Department of General and Environmental Physiology, University of Bari, Italy

ARTICLE INFO

Article history:
Received 4 March 2008
Revised 10 June 2008
Accepted 3 July 2008
Available online 23 July 2008

Keywords:
Muscular dystrophy
Gentamicin treatment
Exercise
Dystrophin
Aquaporin-4
Histology
Electrophysiology
Calcium homeostasis
Creatine kinase
Nuclear factor-kB

ABSTRACT

Aminoglycosides force read through of premature stop codon mutations and introduce new mutation-specific gene-corrective strategies in Duchenne muscular dystrophy. A chronic treatment with gentamicin (32 mg/kg/daily i.p., 8–12 weeks) was performed in exercised mdx mice with the dual aim to clarify the dependence on dystrophin of the functional, biochemical and histological alterations present in dystrophic muscle and to verify the long term efficiency of small molecule gene-corrective strategies in work-loaded dystrophic muscle. The treatment counteracted the exercise-induced impairment of *in vivo* forelimb strength after 6–8 weeks. We observed an increase in dystrophin expression level in all the fibers, although lower than that observed in normal fibers, and found a concomitant recovery of aquaporin-4 at sarcolemma. A significant reduction in centronucleated fibers, in the area of necrosis and in the percentage of nuclear factor-kB-positive nuclei was observed in gastrocnemious muscle of treated animals. Plasma creatine kinase was reduced by 70%. *Ex vivo*, gentamicin restored membrane ionic conductance in mdx diaphragm and limb muscle fibers. No effects were observed on the altered calcium homeostasis and sarcolemmal calcium permeability, detected by electrophysiological and microspectrofluorimetric approaches. Thus, the maintenance of a partial level of dystrophin is sufficient to reinforce sarcolemmal stability, reducing leakiness, inflammation and fiber damage, while correction of altered calcium homeostasis needs greater expression of dystrophin or direct interventions on the channels involved.

Introduction

Premature stop codon mutations in the dystrophin gene occur in about 15% of boys affected by the severe Duchenne muscular dystrophy (DMD), while is typically observed in the dystrophic mdx mouse, the most widely used animal model of DMD (Sicinski et al., 1989; Hoffman and Dressman, 2001). Premature stop codon mutations lead to the total absence of the subsarcolemmal protein dystrophin, to the disorganization of the dystrophin–glycoprotein complex (DGC) and to the consequent loss of the physical linkage between the intracellular cytoskeleton and the extracellular matrix (Hoffman and Dressman, 2001; Petrof, 2002). This defect accounts for a progressive muscle degeneration and weakness, finally leading to premature death due to respiratory or heart failure (Hoffman and Dressman, 2001). Aminoglycosides can read through premature termination codons, permitting translation of full-length proteins in inherited disorders. Being able to enhance dystrophin expression, aminoglycosides opened the way, along with exon-skipping strategies, to promising mutation-based therapeutic approaches by post-translation gene repair processes (Hoffman and Dressman, 2001; Kapsa et al., 2003). A fourteen day treatment with gentamicin partially restores the expression of dystrophin and preserves muscle function in mdx mice; this mechanism might be shared by other aminoglycosides (Barton-Davis et al., 1999; Arakawa et al., 2003; Yeung et al., 2005). Gentamicin also enhances dystrophin at endothelial level, with an increase in eNOS expression and amelioration of shear stress response (Loufrani et al., 2004). While the use of aminoglycosides in DMD patients is hindered by the low percentage of responders and high toxicity (Wagner et al., 2001; Kimura et al., 2005), newly developed molecules, such as PTC 124, acting with a similar mechanism, are a promising therapy for the restricted subset of DMD patients with stop codon mutations (Hirawat et al., 2007; Welch et al., 2007). However, it is still important to identify drugs able to target the pathological cascade and to slow down the progression of the disease in DMD patients irrespective to the mutation; at the

* Corresponding author. Dipartimento Farmacobiologico, Via Orabona 4, Campus, 70125 Bari, Italy. Fax: +39 080 544 2801.
E-mail address: adeluca@farmbiol.uniba.it (A. De Luca).
Available online on ScienceDirect (www.sciencedirect.com).

0969-9961/$ – see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.nbd.2008.07.009

moment only glucocorticoids have clinical applications, in spite of their high toxicity (Bushby and Griggs, 2007). In dystrophin-deficient muscles, the progressive myofiber injury and necrosis, along with muscle tissue loss, are caused by a cascade of complex and interconnecting events occurring with still debated mechanisms and temporal sequence (Hoffman and Dressman, 2001). A fragility of sarcolemma during contractile stress is a typical hallmark, as also documented by the presence of muscular enzymes into the bloodstream (De Luca et al., 2005; Dudley et al., 2006; Radley and Grounds, 2006). In addition, an early and self-sustained inflammatory response, along with a functional ischemia and oxidative stress, contribute to presentation and progression of the pathology, later aggravated by a progressive fibrosis and a failing regeneration (Rando, 2001; Porter et al., 2002; Chen et al., 2005; Dudley et al., 2006; Andreetta et al., 2006; Gosselin et al., 2007; Pierno et al., 2007). An alteration of calcium homeostasis, that in turn activates necrotic pathways through proteolytic enzymes (Alderton and Steinhardt, 2000; Robert et al., 2001; Gailly et al., 2007), also occurs likely sustained by the increased sarcolemmal influx of calcium ions through a stretch/mechano-sensitive calcium channel (Vandebrouck et al., 2002; Iwata et al., 2003; Fraysse et al., 2004; Yeung et al., 2005; Rolland et al., 2006). In agreement with the mechanical role of dystrophin, all these pathways are aggravated by the amount of work load *in vivo*. In the mdx mouse model, the more dramatic signs are in the respiratory diaphragm, for its continuous activity; accordingly a chronic protocol of exercise aggravates the pathology in limb muscles (Stedman et al., 1991; Granchelli et al., 2000; De Luca et al., 2003). The exercised mdx animals are useful for preclinical drug testing, as they show *in vivo* weakness and the exacerbation of disease-related functional and morphological signs *ex vivo* (Grounds et al., 2008; Fraysse et al., 2004; Radley and Grounds, 2006; Rolland et al., 2006). Drugs targeting disease-related pathways exert beneficial effects on the progression of the pathology. However, most of the tested drugs, even those with a wider mechanism of action, can correct some, but not all of the functional and morphological alterations occurring in dystrophic animals (De Luca et al., 2003, 2005; Burdi et al., 2006; Radley and Grounds, 2006; Pierno et al., 2007). Based on these observations we performed a chronic treatment with gentamicin, which would increase dystrophin presence and possibly DGC into dystrophic fibers, in order to 1) better evaluate the direct or indirect dependence of disease-related pathways and alterations upon the primary defect; 2) mechanically reinforce the sarcolemma during the exercise stress and 3) reduce the activity of the channels involved in calcium entry into dystrophic fibers. To this aim gentamicin at 32 mg/kg was administered for 8–12 weeks in exercised mdx mice and the effects were evaluated both *in vivo* and *ex vivo* on various functional, biochemical and morphological indices of muscle damages.

Materials and methods

All experiments were conducted in accordance with the Italian Guidelines for the use of laboratory animals, which conform with the European Community Directive published in 1986 (86/609/EEC).

In vivo experiments

Animal groups and drug treatment

Male 4–5 week old mdx and wildtype (wt, C57/BL10ScSn) mice (IFFA Credo, France and Jackson Laboratories, USA) were used. Initially, all the animals were weighed and forelimb force measured by means of grip strength meter. Mdx mice undergoing the exercise regimen were subdivided into 10 untreated and 17 gentamicin-treated. The gentamicin treatment (32 mg/kg/daily i.p.) started one day before the beginning of the exercise protocol, and continued until the day of sacrifice. Age and sex matched mdx mice (n=8), taken as sedentary, were left free to move in the cage, without additional exercise and were monitored for *in vivo* and *in vitro* studies at the same time points of exercised counterparts, according to the experimental need. Five untreated and 5 gentamicin-treated wt mice were also used as controls and to verify the occurrence of genotype-specific toxicity. On the basis of previous results (De Luca et al., 2003), no exercise regimen has been considered useful, and therefore ethical to be used, in wt animals for the aim of the study. During the gentamicin treatment, the mdx mice did not show any abnormal behavior or difference in macroscopic vital functions vs. untreated groups.

Exercise protocol and in vivo studies

Untreated and treated mdx mice belonging to the exercised groups underwent a 30 min running on an horizontal treadmill (Columbus Instruments, USA) at 12 meter/min, twice a week, for 8 weeks (Granchelli et al., 2000; De Luca et al., 2003). The training protocol started at the mouse age of 4–5 weeks. Fatigue and the avoidance behavior with respect to exercise were observed in few mdx mice and were not modified by the drug treatment. Every week all the mice (sedentary and exercised) were monitored for body weight and forelimb strength by means of a grip strength meter (Columbus Instruments, USA) (De Luca et al., 2005; Pierno et al., 2007). The force of exercised mice was evaluated before the training section. At the end of the 8th week of exercise/treatment the *ex vivo* experiments were started. Due to the time-limiting nature of many *ex vivo* recordings, a 4 week window was needed to complete all the determinations (end of experiments at the 12th week). For this reason animals were continued to be exercised/treated until the day of sacrifice.

In vitro studies

Muscle preparations

The *ex vivo* experiments were performed on different muscles collected from mice belonging to the various groups after 8–12 weeks of either exercise and/or treatment. Age-matched sedentary mice and wt were used at corresponding time points. Thus the age of the animals at the time of experiment was 12–17 weeks. The animals were anesthetized with 1.2 g/kg urethane and the different hindlimb muscles used according to the specific experimental needs detailed below. Diaphragm (DIA) was rapidly removed, placed in normal physiological solution and rapidly cleaned from connective tissue. The right-half side was used for the electrophysiological recordings.

Electrophysiological recordings by intracellular microelectrodes

Extensor digitorum longus (EDL) muscle and hemi-diaphragm (DIA) were placed in the recording chamber at 30±1 °C and superfused with normal and chloride-free physiological solutions. The normal physiological solution had the following composition (in mM): NaCl 148; KCl 4.5; $CaCl_2$ 2.0; $MgCl_2$ 1.0; $NaHCO_3$ 12.0; NaH_2PO_4 0.44 and glucose 5.55. The chloride-free solution was made by equimolar substitution of methyl sulfate salts for NaCl and KCl and nitrate salts for $CaCl_2$ and $MgCl_2$. The solutions were continuously gassed with 95% O_2 and 5% CO_2 (pH=7.2–7.4). Standard two intracellular microelectrode current clamp method was used to record the transient change in membrane potential (electrotonic potential) in response to a hyperpolarizing square wave current pulse and to calculate the membrane electrical properties of muscle fibers, among which membrane resistance (Rm, in Ω^*cm^2), according to the equation $Rm = 0.2\pi_{calc}\lambda R_{in}$, where λ is the space constant, calculated as the logarithmic decay of electrotonic potential with distance between electrodes, R_{in} is the fiber input resistance, calculated as the ratio between the electrotonic potential and the current intensity and d_{calc} is the calculated fiber diameter ($4R_i\lambda/\pi R_{in}$; R_i=myoplasmic resistivity of 140 and 200 Ω^*cm^2 for EDL and diaphragm muscle, respectively; De Luca et al., 1997, 2003). The total membrane capacitance Cm was also calculated as τ/Rm, where τ is the 84% rise time of the electrotonic potential. The total membrane conductance