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Research article

Effects of (−)-epicatechin on frontal cortex DAPC and dysbindin of the mdx mice

Francisco J. Estrada-Mena[a,1], Alonso Rodriguez[b,1], Patricia Mendoza-Lorenzo[c], Teresa Neri-Gomez[d], Gabriel Manjarrez-Gutierrez[d], Andric C. Perez-Ortiz[a], Rosa Ordonez-Razo[e], Guillermo Ceballos[f], Francisco Villarreal[b], Israel Ramirez-Sanchez[b,f,*]

[a] Biochemistry and Molecular Biology, Universidad Panamericana School of medicine, Mexico City, Mexico
[b] School of Medicine, University of California San Diego, La Jolla, CA, USA
[c] Division Academica de Ciencias Basicas, Unidad Chontalpa, Universidad Juarez Autonoma de Tabasco, Tabasco Mexico
[d] Unidad de Investigacion Medica en Enfermedades Neurologicas, Hospital de Especialidades, Centro Medico Nacional Siglo XXI, IMSS, Mexico
[e] Unidad de Investigacion Medica en Genetica Humana, Hospital de Pediatria, Centro Medico Nacional Siglo XXI, IMSS, Mexico
[f] Seccion de Estudios de Posgrado e Investigacion, Escuela Superior de Medicina, Instituto Politecnico Nacional, Mexico

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ABSTRACT

Introduction: Multiple components of the dystrophin-associated protein complex (DAPC) are expressed in numerous tissues including the brain. Members of the DAPC and dysbindin are abnormally expressed in the brain of Duchenne Muscular Dystrophy (DMD) patients, which has been associated with cognitive impairments. However, little is known about the expression pattern of individual members of the DAPC in animal models of DMD and their relationship with dysbindin.

Methods: Ten mdx mice were randomly allocated into a control and intervention group [(−)-epicatechin (Epi) 1 mg/kg/day for four weeks] and results compared to a wild-type mice. After sacrifice, brain pre-frontal cortices were collected for Western blotting and immunoprecipitation assays, and sagittal sections processed for immunohistochemistry.

Results: Epi promotes a partial recovery of DAPC members [α1-Syntrophin, sarcoglycans (SG), dystrophin 71 (Dp71)], dysbindin, and utrophin protein levels. Epi also appears to restore the association of DAPC between dysbindin, and utrophin with Dp71 and ε-SG. Co-immunostaining evidence increased protein levels of dysbindin, dystrophin, and ε-SG and their colocalization.

Conclusions: Altogether, results suggest that Epi is capable of restoring pre-frontal cortex DAPC and dysbindin levels of mdx mice towards that of healthy brains. The functional implications of such studies warrant further investigation.

1. Introduction

Duchenne muscular dystrophy (DMD) is a disease that arises from alterations in the dystrophin protein. In muscle, dystrophin (Dp427) is a central component of the Dystrophin Associated Protein Complex (DAPC) a multiprotein structure with mechano-signaling and cell strengthening roles that links the extracellular matrix to the cytoskeleton [1–3]. The DAPC is formed by: (i) the dystroglycan complex, composed of α- and β-dystroglycan, (ii) the sarcoglycan (SG) complex (SGC) consisting of α-, β-, γ-, δ-, ε-, ζ-SGs, and sarcospan, and (iii) dystrophin/utrophin, dystrobrevins and the syntrophin protein family [1]. The DAPC can also be assembled with utrophin, the autosomal homologue of dystrophin, as observed early during development and soon after birth [4,5]. Besides muscle, multiple DAP-like complexes are expressed in organs such as the brain. The X-linked DMD is a disease that is also associated with cognitive impairment [6]. Full-length dystrophin (Dp427) is present in the cerebral cortex, hippocampus and cerebellum, and is associated with dystroglycan, as well as dystrobrevin and syntrophins [7–11]. Several dystrophin isoforms are generated by alternate intragenic promoter processing producing short variants such

Abbreviations: DMD, Duchenne muscular dystrophy; DAPC, dystrophin-associated protein complex; Dp, dystrophin; Epi, (−)-epicatechin; GABAARs, γ-aminobutyric acid type A receptors-containing synapses; LGMD, limb girdle muscular dystrophy; mdx, dystrophin deficient mouse model; SG, sarcoglycan; SGC, sarcoglycan protein complex; wt, wild type

* Corresponding author. Present address: Seccion de Estudios de Posgrado e Investigacion, Escuela Superior de Medicina del Instituto Politecnico Nacional, Plan de San Luis y Diaz Miron s/n, Colonia Casco de Santo Tomas, Delegacion Miguel Hidalgo, C.P. 11340, Ciudad de Mexico, Mexico.
E-mail address: israel.ramirez14@hotmail.com (I. Ramirez-Sanchez).
[1] Share First author.

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as Dp260, Dp140, Dp116 and Dp71/Dp40. Dp71 is the main isoform expressed in brain and is found in both neurons and glia [12]. Interestingly, dystrophin mutations involving Dp427, Dp140 and Dp71 are frequently associated with mental retardation in DMD patients [13,14].

Many studies have identified brain anatomical and biochemical abnormalities in DMD patients and in the dystrophin-deficient mdx mouse, which is a murine model for DMD [15,16]. Studies have shown that the DAPC, contributes to the establishment and maintenance of γ-aminobutyric acid type A receptors-containing synapses ($GABA_ARs$) in the cerebellum and hippocampus [11,17].

The dysbindin-1 protein, encoded by the DNTBP1 gene, was discovered in the search for dystrophin protein interactions. Dysbindin disruptions have been proposed as contributors to cognitive deficits observed in patients with Duchenne and Becker muscular dystrophy [18]. Dysbindin is associated with GABAergic and glutamatergic synapses both of which play important roles in cognition and are expressed in the pre-frontal cortex. However, no studies have examined the changes in brain dysbindin protein levels or their associations with DAPC members in DMD patients or in mdx mice, which prompted us to study the dysbindin in the animal model.

SG complex subunits are known to be expressed in the human cerebral cortex, both in large neurons and astrocytes [19]. It has been reported that ε-SG is expressed in multiple brain regions, including cerebellar Purkinje cells and dopaminergic neurons of the substantia nigra and ventral tegmental area [20,21]. However, there is no information available regarding the effects of mutations in SG members on brain function with the exception of ε-SG, whose mutation has been related with the presence of myoclonus dystonia syndrome [22] and psychiatric disorders [23].

We previously demonstrated the beneficial effect of treatment with the cacao flavonoid (−)-epicatechin (Epi) on mitochondrial biogenesis/function, oxidative stress and muscle function in a mouse model of limb girdle muscular dystrophy (LGMD)-2F (δ-SG null) [24]. We also reported on positive effects of Epi-rich cocoa supplementation for 3 months on skeletal muscle DAPC protein levels in patients with heart failure and type 2 diabetes [25].

These findings prompted us to examine the effects of Epi on brain DAPC members in mdx mice. Herein, we report on the beneficial effects of Epi on DAPC members, α1-Syntrophin, SGC, Dp71, dysbindin, and utrophin protein levels and molecular interactions in the pre-frontal cortex of these animals.

2. Material and methods

2.1. Animal model

Wild type and mdx mice were purchased from Jackson Laboratories (Bar Harbor, ME). All procedures were performed in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) and the U.S. Guidelines for the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources (http://www.nal.usda.gov/awic/pubs/noawicpubs/careuse.htm).

2.2. Experimental design and approach

We examined the effects of Epi on the expression of DAPC and dysbindin in brain pre-frontal cortex of 6 month old male C57BL/6J wild type (wt) and mdx mice. Animals were housed and provided with water and regular chow *ad libitum*. We separated our mice into three groups (n = 5 animals/group): 1) wild type (wt), and the rest 10 mdx mice that were randomly allocated into two groups 2) mdx; and 3) mdx-epi. Mice were provided either vehicle (water) (wt and mdx) or Epi (1 mg/kg/day dissolved in water) (mdx-epi) (Sigma-Aldrich, St Louis, MO, USA) by oral gavage once a day for 4 weeks. This dose was chosen based on previous studies[26]. At the end of treatment, animals were sacrificed by decapitation during isofluorane anesthesia and the brain tissue was collected. The brain pre-frontal cortex was isolated and frozen in liquid nitrogen for Western blotting (WB) and immunoprecipitation assays (IP). For immunohistochemistry, sections of pre-frontal cortex were embedded in glycerol based media (Tissue-Tec) and frozen using liquid nitrogen-isopentane. Sagittal sections (5 μm thickness) from comparable regions of the pre-frontal cortex were obtained and mounted onto microscope slides.

2.3. Western blotting

Approximately 10 mg of brain pre-frontal cortices were homogenized using a polytron in 150 μl of lysis buffer (1% Triton X-100, 20 mM Tris, 140 mM NaCl, 2 mM EDTA, 0.1% SDS) with protease and phosphatase inhibitor cocktails (P2714 and P2850; Sigma-Aldrich), supplemented with 0.15 mM PMSF, 5 mM Na_3VO_4, and 3 mM NaF. Homogenates were sonicated for 15 min at 4 °C and centrifuged at 12,000*g* for 10 min. Total protein content was measured in the supernatant with the Bradford method. A total of 40 μg of protein were loaded onto a 4–15% precast polyacrylamide gel (Bio-Rad), and electrotransferred to a poly vinylidene difluoride membrane using a semidry system. Membranes were incubated for 1 h in blocking solution (5% nonfat dry milk in NaCl/Tris plus 0.1% Tween-20) and then over night at 4 °C with primary antibodies [27]. To examine SG complex proteins, antibodies against α, β, γ, δ, ε(GeneTex Inc. Irvine, CA, USA), were used. To examine DAPC, antibodies against α-1 syntrophin, dysbindin, α, β, γ, δ SG's (GeneTex Inc. Irvine, CA, USA), utrophin (N-terminus [N-19]), dystrophin (C-terminus [C-20]) (Santa Cruz Biotechnologies, Santa Cruz, CA, USA), were used. GAPDH (Cell Signaling Technology, Inc.) was used as a loading control. Primary antibodies were diluted 1: 250–1000 in blocking solution. After primary antibody incubation the membranes were washed (3 × 5 min each) in NaCl/Tris plus 0.1% Tween- 20 (T-TBS), and incubated for 1 h at room temperature with specific horseradish peroxidase (HRP)-conjugated secondary antibodies. Membranes were again washed 3 times T-TBS, and the immunoblots developed with an ECL Plus detection kit (Amersham-GE, Pittsburgh, PA, USA). Band intensities were digitally quantified with IMAGE J (http://www.nih.gov).

2.4. Immunoprecipitation

IP assays were performed as described previously [27]. Briefly, 15 mg of pre-frontal cortex tissue were lysed with 100 μl of non-denaturing extraction buffer (0.5%, Triton X-100, 50 mmol/l Tris·HCl, pH 7.4, 0.15 mol/l NaCl, and 0.5 mmol/l EDTA) and supplemented with protease and phosphatase inhibitor cocktail, plus 1 mmol/l PMSF, 2 mmol/l Na_3VO_4, and 1 mmol/l NaF. Homogenates were incubated on ice for 15 min with gentle shaking, then centrifuged (10 min) at 12,000*g* at 4 °C. A total of 0.5 mg protein was precleared by adding 1 μg of normal rabbit IgG control and 20 μl prot-G/A-agarose and mixed for 30 min (4 °C), subsequent centrifugation at 12,000*g* for 10 min at 4 °C was performed. The supernatant was recovered and incubated overnight at 4 °C under mild agitation with 3 μg of IP anti ε-SG antibody (Santa Cruz). Twenty microliters of protein G/A-agarose were added and the mixture was incubated at 4 °C for 3 h with shaking. The IP mixture was centrifuged at 12,000*g* for 15 min at 4 °C, and the supernatant was recovered and stored at 4 °C for further purposes. The pellet was washed 3 times with extraction buffer and centrifuged at 12,000*g* for 15 min at 4 °C. The IP proteins in the pellet and those remaining in the supernatant were applied to a 4%–15% gradient SDS-PAGE for WB. The assay was carried out at least 3 times with the IP antibody.

2.5. Immunofluorescence

Pre-frontal cortex tissue sections were mounted and processed for antigen retrieval according to the heat-induced procedure (Declere, Biosciences). Tissue sections were blocked with 10% goat serum and