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The increased ecto-5′-nucleotidase activity in muscle, heart and liver of laminin α2-deficient mice is not caused by an elevation in the mRNA content

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Abstract

We have previously shown that mouse muscle and liver contain catalytically active and inactive ecto-5′-nucleotidase (eNT) variants and that eNT activity in these tissues increases in laminin α2 (merosin)-deficient *Lama2dy* mice. These results prompted us to study whether: (1) the increase of eNT activity depends on the change in the content of merosin between healthy and dystrophic organs; (2) the active and inactive eNT variants arise from the same or distinct mRNAs; (3) the enhancement of the activity is caused by an increase in the eNT mRNA content. Compared to healthy organs, the activity in dystrophic organs increased four-fold in muscle, 1.7-fold in liver, 1.4-fold in heart and not at all in kidney and lung. The level of immunolabelled eNT protein per unit of activity suggested a similar ratio of inactive to active eNT in healthy liver, kidney, heart and muscle, which increased greatly in lung. The size of the eNT subunit in liver, kidney, heart and muscle (72 kDa) decreased to 66 kDa in lung. The identification of a single RT-PCR product suggested that active and inactive eNT arise from the same mRNA and are generated by a differential post-translational processing. Compared to the content in muscle, the amount of eNT mRNA was 12-fold higher in liver and kidney, eight-fold in heart and five-fold in lung. The relative content of eNT mRNA was unaffected by the deficiency of merosin.

Keywords: CD73; Muscular dystrophy; Merosin; Inactive eNT

1. Introduction

Ecto-5′-nucleotidase (eNT, CD73, E.C. 3.1.3.5) hydrolyzes nucleosides 5′-monophosphate to nucleosides, thus controlling the final step of the enzyme cascade that converts extracellular ATP into adenosine (Vollmayer et al., 2001). Adenosine can cross the cell membrane and be used for purine salvage; alternatively, adenosine can produce physiological responses by interacting with purinergic P1 receptors (Chen, Li, & Zou, 2001; de Jong, de Jonge, Keijzer, & Bradamante, 2000; Narravula, Lennon, Mueller, & Colgan, 2000). Beside its hydrolytic action, experimental evidence suggests that eNT plays functions unrelated to catalysis. Through non-catalytic actions, eNT possibly participates in neural development (Braun & Zimmermann, 1998), cell-matrix adhesion phenomena (Paddenberg et al., 1998; Stochaj, Dieckhoff, Mollenhauer, Cramer, & Mannherz, 1989) and signal transduction pathways (Resta et al., 1993).

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From the structural point of view, eNT is a glycoprotein which occurs in tissues and body fluids in a dimeric state with non-covalently bound subunits (Martínez-Martínez et al., 2000). In tissues, eNT resides on the extracellular side of the plasma membrane, where it is anchored by glycosylphosphatidylinositol (GPI) residues (Zimmermann, 1992).

Although laminin-2 is the principal laminin variant in striated muscle, it also occurs, although to a lower extent, in non-muscular tissues, such as liver, kidney and lung (Ringelmann et al., 1999; Sasaki, Giltay, Talts, Timpl, & Talts, 2002). In muscle, laminin-2 connects the basal lamina with the dystrophin-glycoprotein complex (DGC), which bridges the sarcolemma and links the extracellular matrix with the actin cytoskeleton (Sasaki et al., 2002). Various classes of muscular dystrophies arise from the anomalous expression of specific DGC components (Cohn & Campbell, 2000).

Laminin-2 is a heterotrimer which consists of α2 (merosin), β1 and γ1 chains. About half of the cases of classical congenital muscular dystrophy (CMD) arise from mutations of merosin. The homozygous merosin-deficient *Lama2dy* mouse displays a dystrophic phenotype, skeletal muscle pathology and peripheral neuropathy (Tomé, 1999) which makes it a suitable model for investigating the pathological features associated with CMD (Allamand & Campbell, 2000; Tomé, 1999). In our search for the possible pathological signs that the absence of merosin produces in skeletal muscle, we have previously reported that eNT activity increases nearly four-fold in muscle of *Lama2dy* mice. In spite of the great increase in activity, Western blots of samples of healthy and dystrophic muscle, with the same units of eNT activity, revealed that the content of immunolabelled eNT protein is 2–3 times higher in control muscle (García-Ayllón, Campoy, Vidal, & Muñoz-Delgado, 2001). The greater amount of eNT protein per unit of activity in control than in pathological muscle strongly supported the existence of catalytically inactive eNT in muscle, while the greater activity in the diseased muscle suggested that the fraction of active eNT was increased by dystrophy. Finally, the similar content of eNT protein in healthy and dystrophic muscle led us to conclude that dystrophy disrupted the normal balance between active and inactive eNT, the disease driving the production of active eNT at the expense of inactive eNT (García-Ayllón et al., 2001). The attempts to ascertain whether the production of inactive eNT is restricted to muscle or is extended to other tissues revealed the presence of inactive eNT in liver (Morote-García, Carcía-Ayllón, Campoy, Vidal, & Muñoz-Delgado, 2004). As in the case of muscle, the amount of eNT protein per unit of activity in liver was also reduced by dystrophy (Morote-García et al., 2004).

Despite the identification of a single eNT mRNA in mouse organs and cells (Resta et al., 1993), the occurrence of nine exons in the gene raises the possibility that active and inactive eNT are encoded by alternatively spliced mRNAs. The aim of this research was to explore the above possibility, to compare also the levels of eNT mRNA, eNT protein and activity in mouse tissues and to investigate whether the levels are affected by the deficiency of merosin.

2. Materials and methods

2.1. Materials

Benzamidine, α,β-methylene-ADP, sodium deoxycholate, phenylmethylsulfonyl fluoride (PMSF), *N*-[2-hidroxyethyl]-piperazine-*N*′-[2-ethanesulfonic acid] (HEPES), Fiske-Subbarow reagent, concanavalin A-agarose (Con A-agarose), α-methyl-D-mannoside (methyl-mannoside), AMP-agarose, ethidium bromide, diethyl pyrocarbonate (DEPC), deoxyribonuclease I (DNase I), alkaline phosphatase anti-rabbit IgG, 5-bromo-4-chloro-3-indolylphosphate and nitroblue tetrazolium (BCIP/NBT) were all purchased from Sigma (St. Louis, USA). AMP sodium salt was purchased from Fluka (Switzerland), Triton X-100 from Merck (Germany), and ultra pure DNA grade agarose from Bio-Rad (Hercules, USA). Trizol reagent, Moloney murine leukaemia virus (M-MLV) reverse transcriptase recombinant from *E. coli* and specific eNT primers were obtained from Life Technologies (USA). Random decamers and the QuantumRNA 18S Internal Standards kit were from Ambion (United Kingdom). Hot Master *Taq* DNA polymerase and dNTPs were purchased from Eppendorf (Germany). A rabbit antiserum against eNT from bull seminal plasma was kindly provided by Prof. C. Fini of the University of Perugia.

2.2. Animals and tissue isolation

Phenotypically normal *Lama2* (+/?) and dystrophic (merosin-negative and dystrophin-positive) *Lama2* (*dy*/*dy*) B6.129P1 mice were provided by Jackson Laboratories (Bar Harbor, USA). They were bred and maintained at the animal house of the University of Murcia according to ethical animal care guidelines. Animals, 3–4 months old and without sex distinction, were ether anaesthetized before opening the thoracic cavity. After perfusion of heart with 5.4 mM EDTA, 154 mM NaCl, pH 7.4, hind limb and back muscle, liver,