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ScienceDirect

Neuromuscular Disorders 25 (2015) 888–897

www.elsevier.com/locate/nmd

Fast-twitch skeletal muscle fiber adaptation to SERCA1 deficiency in a Dutch Improved Red and White calf pseudomyotonia case

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Received 17 March 2015; received in revised form 19 August 2015; accepted 29 August 2015

Abstract

Missense mutations in *ATP2A1* gene, encoding SERCA1 protein, cause a muscle disorder designed as congenital pseudomyotonia (PMT) in Chianina and Romagnola cattle or congenital muscular dystonia1 (CMD1) in Belgian Blue cattle. Although PMT is not life-threatening, CMD1 affected calves usually die within a few weeks of age as a result of respiratory complication. We have recently described a muscular disorder in a double muscle Dutch Improved Red and White cross-breed calf. Mutation analysis revealed an *ATP2A1* mutation identical to that described in CMD1, even though clinical phenotype was quite similar to that of PMT. Here, we provide evidence for a deficiency of mutated SERCA1 in PMT affected muscles of Dutch Improved Red and White calf, but not of its mRNA. The reduced expression of SERCA1 is selective and not compensated by the SERCA2 isoform. By contrast, pathological muscles are characterized by a broad distribution of mitochondrial markers in all fiber types, not related to intrinsic features of double muscle phenotype and by an increased expression of sarcolemmal calcium extrusion pump. Calcium removal mechanisms, operating in muscle fibers as compensatory response aimed at lowering excessive cytoplasmic calcium concentration caused by SERCA1 deficiency, could explain the difference in severity of clinical signs.

Keywords: Cattle congenital pseudomyotonia; Skeletal muscle disease; Sarcoplasmic reticulum (SR); Sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA1); Calcium homeostasis; Human Brody disease

1. Introduction

The sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA), the main protein component of the non-junctional sarcoplasmic reticulum (SR) [1], transports two Ca^{2+} ions from the cytosol into the SR lumen at the expense of the hydrolysis of one ATP molecule. This initiates muscle relaxation.

Three genes (*ATP2A1–3*) encode the SERCA isoforms, which are differentially expressed in a tissue-dependent manner [2]: *ATP2A1* gene encodes SERCA1 isoform, expressed in adult fast-twitch (type 2) skeletal muscle fibers, *ATP2A2* encodes the isoform shared by cardiac and slow-twitch (type 1) skeletal muscle fibers (SERCA2a) [3]. Finally, *ATP2A3* encodes SERCA3 isoform, mostly found in non-muscle cells.

In cattle, muscular diseases associated with *ATP2A1* gene mutations were described in the Chianina and Romagnola breeds [4,5], and in the Belgian Blue breed [6]. These were termed congenital pseudomyotonia (PMT) (in the first two breeds) or congenital muscular dystonia1 (CMD1) (in the Belgian Blue breed). The Belgian Blue breed is characterized by increased muscle mass, known as double muscling [7], caused by a mutation in the myostatin gene, a negative regulator of skeletal muscle growth [8].

PMT was described for the first time by Testoni et al. [9] in Chianina cattle. Although renowned for its somatic gigantism, the Chianina breed is not a double-muscled phenotype [10]. Chianina cattle PMT, transmitted as an autosomal recessive trait [4], was characterized by an impairment of muscle relaxation induced by exercise. When animals are stimulated into intense physical activity, muscles stiffen temporarily, resulting in a rigid gait. If the exercise is prolonged, the contracture hampers movement and the affected animals consequently fall down. After a few seconds of rest, the stiffness wanes and the animals

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http://dx.doi.org/10.1016/j.nmd.2015.08.010
0960-8966/

regain the ability to get up and move. The missense mutation in *ATP2A1* gene causing Chianina cattle PMT [4] replaces an Arg at position 164 by a His (R164H). Due to similarity of clinical signs, cattle PMT is considered the true counterpart of human Brody disease [11].

In parallel, CMD1 was described in Belgian Blue breed [6]. While PMT is not life-threatening, CMD1 affected calves showed impaired swallowing, decreased growth rate and usually die within a few weeks of birth as a result of respiratory complications. In CMD1, the *ATP2A1* gene missense mutation leads to an Arg559Cys (R559C) substitution in the highly conserved cytoplasmic N domain of SERCA1 protein. All Belgian Blue CMD1 cases have been found to be homozygous for *ATP2A1* gene mutation [6].

Recently, we reported a single case of PMT in the Dutch Improved Red and White (VRB) cross-breed calf [12]. The calf, a double-muscled phenotype, was the offspring of a pure-breed VRB sire and a cross-breed dam (Piedmontese × VRB). Piedmontese is another well-known double-muscled breed [8,10]. It has been suggested that the myostatin mutation was introduced in the VRB breed from the Belgian Blue population during the selection for double-muscled modern breeds [13]. Similar to Chianina cattle, the clinical phenotype of the VRB PMT case was characterized by a generalized muscle stiffness exacerbated by physical activity. Nevertheless, DNA sequencing revealed a homozygous genotype for a missense *ATP2A1* gene mutation identical to that described in Belgian Blue cattle, which replaces the Arg at position 559 by a Cys in the SERCA1 protein [12].

Although the SERCA1 mutation in VRB and Belgian Blue clinical cases is the same, a marked discrepancy in the severity of pathology has been described between the two breeds. We concluded that PMT and CMD1 refer to the same congenital condition that may present with different phenotypes depending on the breed [12].

The purpose of the present study was to investigate the muscle specimens from this single case of VRB cross-breed calf PMT in order to characterize the phenotype at both biochemical and immunohistochemical levels.

2. Materials and methods

2.1. Animals

Biopsies of semimembranosus muscle were obtained during diagnostic procedures from a PMT-affected VRB cross-breed calf and two double muscling clinically healthy VRB control animals of the same gender and age, as described in our previous work [12]. The national and institutional guidelines for the care and use of animals were followed and all procedures were approved by the local Institutional Animal Care and Use Committee. Semimembranosus muscle specimens were collected from three healthy pure-breed Italian Piedmontese cattle immediately after slaughter.

2.2. SERCA1 construct and site-directed mutagenesis

The full-length adult bovine SERCA1 cDNA was synthetically generated by Eurofins (Germany) based on the published database sequence of *Bos taurus* (cow) SERCA1 mRNA (GenBank cDNA clone MGC: 140007). Restriction sites *Hind*III and *Not*I were added upstream of the ATG and downstream of the STOP codon, respectively, to allow the fragment cloning in the pcDNA3.1 expression vector. The R559C SERCA1 substitution was generated by using the QuikChange site-directed mutagenesis kit (Stratagene), according to the manufacturer's specifications [14]. The mutagenic primers (Eurofins, Germany) (forward 5′-CCGGG ACACCCTG**T**GCTGCCTGGCGCTGGC and reverse 5′-GCCA GCGCCAGGCAGC**A**CAGGGTGTCCCGG), annealing in the region where the mutation has to be introduced, were designed with a mismatch centered to the nucleotide 1675 (bold, underlined letters). The construct was verified by sequencing.

2.3. Histochemistry and immunocytochemistry

Muscle samples were frozen in cold isopentane. Transverse sections (8 μm) were cut in a cryostat. Serial sections were stained for hematoxylin and eosin (H&E), α-naphthyl acetate for esterase, cytochrome oxidase (COX), succinic dehydrogenase (SDH) and myofibrillar adenosine triphosphatase (m-ATPase). Serial sections were incubated with the following primary antibodies: mouse-monoclonal to SERCA1a (dilution 1:500, Biomol, Plymouth Meeting); rabbit-polyclonal to neonatal myosin heavy chain isoform [15], rabbit-polyclonal to mitochondrial preprotein traslocases of the outer membrane 20 (Tom20, dilution 1:100, Santa Cruz Biotechnology, Texas, USA). Sections were then incubated with the appropriate secondary antibody either conjugated with TRITC (tetramethylrhodamine isothiocyanate) (Dako, Milano, Italy) or with biotin (Sigma-Aldrich). In the latter case, the reaction was revealed with the Envision method (Vector, Burlingame, USA) and visualized with Olympus Vanox AH-3 bright field microscope. Confocal microscopy was performed using a Leica TCS-SP2 confocal laser scanning microscope.

Measurements of fiber areas were recorded using software Adobe Photoshop CS4 on COX stained slide images (magnification 10×). Fibers were chosen randomly among 20 images per sample and a total of 200 light labeled fibers and 200 dark labeled fibers per animal were measured.

2.4. Preparative procedures

A crude microsomal fraction enriched in content of sarcoplasmic reticulum (SR) membranes was isolated from muscle biopsies. Muscles were homogenized in 10 mM HEPES, pH 7.4, 20 mM KCl containing protease inhibitors 1 μg/ml leupeptin and 100 μM phenylmethylsulfonyl fluoride as described [11,16]. The myofibrils were sedimented by centrifugation at 650 × g for 10 min at 4 °C. The crude SR fraction was obtained from the previous supernatant by ultracentrifugation at 120,000 × g for 90 min at 4 °C. Membrane fractions were resuspended in 0.3 M sucrose, 5 mM imidazole, pH 7.4 containing 1 μg/ml leupeptin and 100 μM phenylmethylsulfonyl fluoride and stored at −80 °C.

Total muscle homogenates were obtained from roughly 100 mg of muscle biopsy by solubilization in lysis buffer (62.5 mM Tris–HCl (pH 6.8), 10% glycerol, 2.3% sodium dodecyl sulfate and protease inhibitors). Cell debris were removed by centrifugation at 16,000 × g for 10 min at 4 °C. Protein concentration was determined by the method of Lowry et al. [17] using bovine serum albumin as a standard.