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MALDI reveals membrane lipid profile reversion in MDX mice

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ABSTRACT

Duchenne muscular dystrophy (DMD), the most common and severe X-linked myopathy, is characterized by the lack of dystrophin, a sub-sarcolemmal protein necessary for normal muscle functions. In a previous study of the lipid content of skeletal muscles of dystrophic (*mdx*) mice, the animal model for DMD, by *in situ* Matrix-Assisted Laser Desorption-Ionization Mass Spectrometry (MALDI-MS), an inversion of the phosphatidylcholine PC34:2/PC34:1 ion peaks intensity ratio was observed between destructured (abnormal fiber morphology) and structured (normal fiber morphology). A possible treatment for this dramatic disease is to introduce an exogenous nitric oxide (NO) donor into the organism, leading to an increase of utrophin and a regression of the dystrophic phenotype. In the present work, after confirmation by tandem mass spectrometry of the structure of these two phospholipids, their intensity ratio inversion was used to evidence a restoration of membrane lipid composition very similar to those of wild-type mice after the treatment of *mdx* mice with molsidomine, a NO donor. This was associated with the observation by immunohistology of an increase of the regeneration process in the mice.

Introduction

Duchenne muscular dystrophy (DMD) is an X-linked neuromuscular disorder which affects one boy over 3500. DMD is caused by mutations of a gene located on the Xp21 locus coding for the dystrophin, a sub-sarcolemmal protein. This intracellular protein supports the muscle cell structure with connection between the cytoskeleton and the extracellular matrix of muscle fibers through interaction with the dystrophin-associated glycoproteins complex (DGC) (Blake et al., 2002). The lack of this molecule induces a weakness of myofibers due to mechanical muscle contraction, leading to dramatic muscle deterioration. Beyond this principal cause, Ca^{2+} leakage (Gillis, 1996), oxidative stress (Niebrój-Dobosz and Hausmanowa-Petrusewicz, 2005), inflammatory response (Porter et al., 2002), and fat infiltration are observed in human dystrophic muscle. The first signs of this disease appear at the age of 3 or 4 years and the life expectancy rarely goes past 20 years old.

The three main therapeutic approaches are gene therapies, cells therapies and pharmacological treatments (Voisin and De la Porte, 2004) At this time, there is no effective therapy for the DMD patients but a lot of therapeutic tests are under progress on *mdx* mice, the most widely used animal model (Bulfield et al., 1984). *Mdx* mouse shows an efficient regeneration process (Pastoret and Sebille, 1995), leading to less destructured areas in muscle tissue than in human. This provides an attenuated dystrophic phenotype, with a quasi normal life expectancy for *mdx* mice.

Matrix-Assisted Laser Desorption-Ionization Mass Spectrometry (MALDI-MS) (Karas et al., 1987) is a particularly attractive mass spectrometry method for direct profiling of tissue sections (Stoeckli et al., 2001). It can be used to find and eventually localize metabolites or potential biomarkers, so it is a powerful method to analyze processes of physiological importance. Mass spectrometry profiling allows direct *in situ* analyses of structured and destructured areas from the same muscle tissue sections. Indeed, since destructured cells represent a small quantity of fibers of the total muscular mass, the specific information corresponding to these regions can be hidden by the major part of the muscle when *in toto* analysis is performed (Blake et al., 2002). Mass spectrometry is the only method which can be used to identify various biological molecules on entire tissue sections with high resolution and sensitivity in a minimum of time.

A precedent study performed by MALDI-MS profiling revealed changes in phospholipid composition within dystrophic mouse leg sections (Touboul et al., 2004). This analysis indicated an inversion of the ratio of the m/z 758.6 (PC 34:2) ion intensity over the m/z 760.6 (PC 34:1) ion intensity between control areas (healthy muscle and structured areas from dystrophic muscles) and destructured areas (from dystrophic muscles). On the other hand, cell cultures were

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performed and the same intensity ratio inversion occurred during the differentiation from myoblastes to myotubes. As this process is obtained by the fusion of the myoblastes membrane, mainly composed of phospholipids, it was thus suggested that this ratio could be a marker of the regenerating process of muscle cells. This variation was recently confirmed by cluster-TOF-SIMS (Time-of-flight Secondary Ion Mass Spectrometry) imaging on human dystrophic tissue sections (Tahallah et al., 2008).

Among the strategies proposed, the upregulation of utrophin to replace defectious dystrophin is being developed (Squire et al., 2002). Utrophin is a protein presenting an 80% homology with dystrophin (Love et al., 1989). Both are found to have similar cellular functions. At the early stage of muscle formation, utrophin is expressed all around immature cells, and is progressively substituted by dystrophin in adult fibers (Karpati et al., 1993). Utrophin only subsists in neuromuscular junctions of adult cells and, like dystrophin, interacts with the DGC. The muscular isoform of nitric oxide synthase (NOS) interacts with syntrophin, a protein of the DGC (Brenman et al., 1996). NOS catalyzes the conversion of L-arginine into nitric oxide (NO). Previous studies demonstrate an increase of utrophin labeling and improvement of dystrophic phenotype, mainly a decrease of necrotic surface and a diminution of creatine kinase release in the serum and of inflammation, as consequences of improved NO production (Barton et al., 2005; Chaubourt et al., 1999; Hnia et al., 2008; Ségalat et al., 2005; Voisin et al., 2005) via the injection of L-arginine or of molsidomine, an NO donor. Molsidomine, commercialized as Corvasal®, a coronary vasodilator indicated for angina pectoris is metabolized at the hepatic level into linsidomine (SIN-1), an unstable metabolite in biological medium releasing a NO radical.

On the basis of these previous results, we decided to use a MALDI-MS profiling strategy and more especially the intensity ratio inversion between the two phosphatidylcholine ions, after having confirmed their identification by tandem mass spectrometry, to study the effect of molsidomine treatment on dystrophic muscle membranes.

Materials and methods

Materials

Three groups of mice ($n=4$ for each group) were treated in agreement with the guidelines of the French Council for the care and use of laboratory animals. Wild-type mice (C57BL10) and *mdx* mice were purchased from Charles River laboratories (L'Arbresle, France) and housed in the Cellular and Molecular Neurobiology Laboratory (INAF, Institut de Neurobiologie Alfred Fessard, Gif-sur-Yvette, France). Four *mdx* mice were injected intra-peritoneally with 10 mg/kg/d of molsidomine (Sigma-Aldrich, St Quentin Fallavier, France) five days out of seven for six weeks and four others with physiological serum. C57BL10 and *mdx* mice were 11–12 weeks old when they were sacrificed. The MALDI matrix α-cyano-4-hydroxy-cinnamic acid (CHCA) and 2,5-dihydroxybenzoic acid (DHB) were purchased from Sigma (Sigma-Aldrich, St Quentin Fallavier, France). A peptide mixture named Pepmix5 (Laser Biolabs, Sophia-Antipolis, France) was used for the calibration of MALDI-TOF mass spectra. This contains Bradykinin [1–5] m/z 573.31, Bradykinin [1–7] m/z 757.40, Bradykinin m/z 1060.57, Angiotensin I m/z 1296.69 and Neurotensin m/z 1672.92.

Sample preparation

Mice were anesthetized by intra-peritoneal injection of pentobarbital 8% and sacrificed by cervical dislocation to minimize tissue alteration. Hind limbs were dissected, frozen in isopentane cooled by liquid nitrogen to prevent cell disruption and stored at −80 °C until cutting. To prevent contamination by the OCT (optimized cutting temperature) glue, tissues were embedded in a tragacanth gum (Prolabo, VWR International, France) prior isopentane freezing. For MALDI profiling, 12 µm thick tissue sections were prepared at −20 °C with a cryostat (CM3050S Leica Microsystems SA, Rueil-Malmaison, France) whereas 7 µm thick mice leg sections were needed for histology. The tissue slides were immediately deposited on stainless steel MALDI plates for the MALDI profiling studies and on gold coated glass plates for histology.

Masson's trichrome staining

Cryostat sections (7 µm) of muscles were stained with Masson's trichrome to visualize connective tissue and muscle fibers in pink and collagen in blue (Sigma kit #HT15) and then observed using a Leica DM RXA2 microscope (Leica Microsystemes, Germany). The sections were photographed using a CoolSNAP camera (Roper Scientific, USA) and Openlab acquisition software (Improvision, UK).

Serum creatine kinase (CK) determination

Blood samples were taken from the hearts of anaesthetized mice immediately before sacrifice. Activities of serum CK were determined using a Biomerieux kit (enzyline CK NAC optimized 10).

MALDI-TOF/TOF MS/MS identifications

Five sections (thickness 50 µm) from wild-type mouse legs were incubated during 2 days in 100% chloroform at 4 °C. The analyte was diluted at 1/10 in 2,5-dihydroxybenzoic acid matrix (10 mg mL^{-1} MeOH, 0.1% TFA) and the solutions were manually deposited on a stainless steel plate. The MS/MS spectra of m/z 758.6 and m/z 760.6 ions were recorded using the 4800 MALDI-TOF–TOF mass spectrometer (mode MS/MS positive, 2 kV, CID on with air as collision gas and the metastable ion suppression was not selected). A peptide mixture named calibration mixture 1 (Applied Biosystems, Les Ulis, France) was used for the calibration of MALDI-TOF MS/MS mass spectra at a dilution of 1/10 in 2,5-dihydroxybenzoic acid matrix. This contains des-Arg-Bradykinin m/z 903.47, Angiotensin I m/z 1295.69, Glu-Fibrinopeptide B m/z 1569.68, Neurotensin m/z 1671.92.

MALDI-TOF MS profiling

Before MALDI-TOF MS profiling, tissue slides were controlled by optical microscopy (Olympus BX51 microscope, Rungis, France) in order to select the destructured or structured areas to be analyzed. The mass spectrometry profiling of tissue sections was performed with a 4800 MALDI-TOF–TOF mass spectrometer (Applied Biosystems, Les Ulis, France). The CHCA matrix used for this study was prepared at a concentration of 20 mg mL^{-1} in V/V 1:1 acetonitrile/water (0.1% TFA), and several micro droplets of the matrix solution were deposited onto the tissue. Three successive droplets at each position, each having a volume of 400 nL. The resulting spot size was always smaller or equal to 300 µm, which is a value always smaller than the size of the destructured areas. The spectra were recorded in reflector mode over the mass-to-charge ratio range m/z 500–1000 (300 shots/spot). External standards were used for the calibration of the spectra (see Materials). Data were subjected to Student's t test for calculation of statistical significance. Means ± SEM are presented throughout, with the level of significance set at $p<0.05$.

Immunohistochemistry

After fixation with cold methanol (+4 °C for 10 min), the cryostat sections were incubated with a specific antibody directed against