Biochimica et Biophysica Acta 1852 (2015) 2722–2731

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

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

Signaling pathways underlying skeletal muscle wasting in experimental pulmonary arterial hypertension

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ARTICLE INFO

Article history:
Received 4 May 2015
Received in revised form 26 September 2015
Accepted 1 October 2015
Available online 9 October 2015

Keywords:
Pulmonary arterial hypertension
Skeletal muscle wasting
Proteolysis
Mitochondrial dysfunction

ABSTRACT

Background: Skeletal muscle wasting contributes to the poor functional status and quality of life of patients with pulmonary arterial hypertension (PAH). The present study aims to characterize the molecular mechanism underlying skeletal muscle wasting in experimental PAH induced by monocrotaline (MCT).
Methods: Male Wistar rats were randomly injected with saline solution (CONT; n = 10) or MCT (MCT; 60 mg/kg, s.c.; n = 15). After 4 weeks of MCT or vehicle administration, animals were anesthetized and submitted to right ventricular (RV) hemodynamic evaluation. Blood and *gastrocnemius* samples were collected and stored for analysis.
Results: MCT group developed PAH (70% increase in RV peak systolic pressure) RV dysfunction (increased end-diastolic pressure and Tau), and body and muscle wasting (reduction of 20%, 16% and 30% on body weight, *gastrocnemius* mass and fiber cross sectional area, respectively). Muscle atrophy was associated with a decrease in type I MHC. Circulating (C reactive protein, myostatin and IL-1beta) and local catabolic markers (MAFbx/atrogin-1, protease activity) were increased in MCT animals, while Akt/mTOR pathway was preserved. Mitochondria isolated from *gastrocnemius* of MCT animals showed decreased activity of ATP synthase, lower levels of Tfam, accumulation of oxidatively modified proteins together with reduced levels of paraplegin.
Conclusions: Our data suggests an anabolic/catabolic imbalance in *gastrocnemius* from MCT-induced PAH rats. Accumulation of dysfunctional mitochondria due to the inefficiency of protein quality control systems to eliminate damaged proteins could also contribute to muscle atrophy in PAH.

1. Introduction

Exercise intolerance experienced by patients with pulmonary arterial hypertension (PAH) is classically attributed to poor functional status secondary to cardiac and respiratory impairment [1,2]. Compelling evidences suggest that PAH patients have intrinsic skeletal muscle abnormalities [3,4], namely: i) decreased type I fibers; ii) increased anaerobic metabolism in skeletal muscles [4]; iii) muscle atrophy; and iv) impaired contractility [5].

Understanding the molecular mechanisms underlying PAH-related muscle cachexia is crucial for the development of effective preventive and/or therapeutic approaches. Increased levels of pro-inflammatory cytokines such as TNF-alpha, IL-1 and IL-6 induce an anabolic/catabolic imbalance in the skeletal muscle [6–12]. The activation of serine/threonine kinase Akt (PKB) and mammalian target of rapamycin (mTOR) [13] is compromised in the atrophic skeletal muscles in both chronic heart failure [14] and cancer [15]. Impairment of mitochondrial quality control (PQC) system [16,17] and accumulation of dysfunctional mitochondria lead to profound disorganization of skeletal muscle [18], and is involved in cancer-induced muscle cachexia [15]. Whether these pathways are present in the wasting muscle in the context of PAH remains unknown.

The main purpose of the present study was to assess the molecular mechanisms underlying muscle wasting in an animal model of PAH induced by monocrotaline (MCT). For this purpose, skeletal muscular morphofunctional features and biochemical markers involved in the regulation of muscle mass were evaluated.

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http://dx.doi.org/10.1016/j.bbadis.2015.10.002
0925-4439/

2. Material and methods

2.1. Animals and experimental design

Housing and experimental treatment of animals were in accordance with the Directive 2010/63/EU. The experiments were performed in compliance with the current national laws (DL 129/92, DL 197/96, P1131/97).

Twenty-five male Wistar rats (age: 5 weeks; weight: 150 g; Charles River Laboratories, Barcelona, Spain) were housed in groups of 5 rats/cage, in a controlled environment at a room temperature of 22 °C with inverted 12:12 h light–dark cycle, with free access to food and water. Rats were randomly divided as follows: CONT (n = 10) injected with saline solution or MCT (n = 15), injected subcutaneously with monocrotaline (MCT) (single dose of 60 mg/kg, s.c., Sigma). Four weeks after MCT or vehicle administration, animals were weighed, submitted to hemodynamic evaluation and then sacrificed under anesthesia by the inhalation of a mixture of 4% of sevoflurane with oxygen. Blood samples (~2 mL) were collected from the inferior vena cava, centrifuged for 5 min at 5000 *g* and an aliquot of serum was obtained and stored at −80 °C for biochemical determination. *Gastrocnemius* muscles were excised, weighed and divided. One piece was immediately processed for light microscopic evaluation and the remaining tissue was separated for MHC isoform quantification and Western blotting analysis of the whole muscle. The other muscle was used for mitochondrial isolation.

2.2. Hemodynamic evaluation

At days 28–29 after MCT or vehicle administration, animals were prepared for right ventricular hemodynamic evaluation. Rats were anesthetized by inhalation with a mixture 4% sevoflurane with oxygen, intubated for mechanical ventilation (TOPO, Kent Scientific). The right jugular vein was cannulated for fluid administration (prewarmed 0.9% NaCl solution) and the heart was exposed by a median sternotomy and the pericardium was widely opened. RV hemodynamic function was measured with conductance catheter (FTS-1912B-8018, Scisense). The catheter was connected to MVP-300 conductance system through interface cable (PCU-2000 MPVS, FC-MR-4, Scisense), coupled to PowerLab16/30 converter (AD Instruments) and a personal computer for data acquisitions. After complete instrumentation, the animal preparation was allowed to stabilize for 15 min. Hemodynamic recordings were made with respiration suspended at the end of expiration under steady-state conditions. Parameters from conductance catheter were recorded at a sampling rate of 1000 Hz and analyzed with Millar conductance data acquisition and analysis software (PVAN3.5).

2.3. Blood tests

Serum albumin, total protein, cholesterol, HDL-cholesterol and triglycerides were measured in duplicate on an AutoAnalyzer (PRESTIGE 24i, Cormay PZ). Serum levels of IL-1 beta were detected by a commercially available ELISA kit, used according to the manufacturer's instructions (R&D). Serum C-reactive protein (CRP) and myostatin levels were assayed by Western blotting as described below. Serum from each animal was assayed in triplicate.

2.4. Morphological analysis

Cubic pieces from *gastrocnemius* muscle (~2 mm^3) were fixed with buffered paraformaldehyde 4% (*v*/*v*) by diffusion during 24 h and subsequently dehydrated with graded ethanol and included in paraffin blocks. Xylene was used in the transition between dehydration and impregnation. Serial cross sections (5 μm of thickness) of paraffin blocks were cut by a microtome and mounted on silane-coated slides. The slides were dewaxed in xylene and hydrated through graded alcohols finishing in phosphate buffered saline solution (pH to 7.2). Deparaffinized sections were stained for hematoxylin-eosin by immersing slides in Mayer's hematoxylin solution for 3–4 min followed by immersion in 1% eosin solution for 7 min, dehydration with graded alcohols through xylene, and mounted with DPX for analysis in a photomicroscope (Zeiss Phomi 3). Photographs of *gastrocnemius* cross sections of all experimental groups were digitalized and analyzed with the NIH ImageJ (Image Processing and Analysis in Java, USA) software. An average of 600 ± 170 fibers were analyzed per group for area quantification.

2.5. Gastrocnemius muscle preparation for biochemical analysis

For the analysis of biochemical markers of anabolic and catabolic pathways, muscles were homogenized at 4 °C in RIPA lysis buffer (1% Nonidet P40, 0.5% sodium deoxycholate, 0.1% SDS in 50 mM NaCl, 20 mM Tris–HCl pH 7.6) containing phosphatase inhibitors (Sigma, catalog numbers P0044 and P5726). Homogenates were clarified by centrifugation at 12,000 ×*g* for 20 min before determination of protein concentration by the colorimetric method "RC DC protein assay" (Bio-Rad) using bovine serum albumin (BSA) as standard. An aliquot of whole muscle homogenate was used for zymography/nLC–MS/MS analysis of proteases.

2.6. Analysis of myosin heavy chain (MHC) isoforms

Gastrocnemius sections were weighed and transferred to a glass homogenizer. A 1:19 ratio of 100 mM phosphate buffer, pH 7.4, containing 0.02% bovine serum albumin was added and muscle sections were thoroughly homogenized with tightly fitted Potter-Elvehjen homogenizer and Teflon pestle. Total protein concentration was spectrophotometrically assayed with the colorimetric method "RC DC protein assay" (Bio-Rad) using bovine serum albumin (BSA) as standard. MHC isoforms were separated by gel electrophoresis following the procedure described by Talmadge and Roy [19]. One microgram of sample from each group studied was applied in the same gel. The stacking gel consisted of 30% glycerol and 4% acrylamide: *N*,*N*′-methylene-bis-acrylamide in the ratio of 50:1, 70 mM Tris (pH 6.7), 4 mM EDTA, and 0.4% sodium dodecyl sulfate (SDS). The separating gels were composed of 30% glycerol, 8% acrylamide-bis (50:1), 0.2 M Tris (pH 8.8), 0.1 M glycine, and 0.4% SDS. Polymerization was initiated with 0.05% *N*,*N*,*N*′,*N*′-tetramethylethylenediamine and 0.1% ammonium persulphate. The gels were run in a Mini-Protean system (Bio-Rad) at 4 °C. The running conditions were 70 V (constant voltage) for 24 h. The gels were stained with Colloidal Coomassie Blue, scanned in Molecular Imager Gel Doc XR+ System (Bio-Rad, Hercules, CA, USA) and optical density analysis of MHC bands was performed using QuantityOne Imaging software (v4.6.3, Bio-Rad).

2.7. Mitochondria isolation from gastrocnemius muscle

Gastrocnemius muscles were also used for the preparation of isolated mitochondria, as previously described [20]. All the procedures were performed on ice or below 4 °C. Briefly, muscles were immediately excised and minced in ice-cold isolation medium containing 100 mM sucrose, 0.1 mM ethylene glycol tetraacetic acid (EGTA), 50 mM Tris–HCl, 100 mM KCl, 1 mM KH_2PO_4, and 0.2% bovine serum albumin (BSA), pH 7.4. Minced blood-free tissue was rinsed and suspended in fresh medium containing 0.2 mg/mL bacterial proteinase (Nagarse E.C.3.4.21.62, type XXVII; Sigma, St. Louis, MO) and stirred for 2 min. The sample was then carefully homogenized with a tightly fitted Potter-Elvehjem homogenizer and a Teflon pestle. After homogenization, Nagarse-free isolation medium was added to the homogenate, which was then centrifuged at 700 ×*g* for 10 min. The resulting supernatant suspension was centrifuged at 10,000 ×*g* for 10 min. The supernatant was decanted, and the pellet was gently resuspended in the isolation medium and centrifuged at 7000 ×*g* for 3 min. The final pellet,