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Hypoxia alters contractile protein homeostasis in L6 myotubes

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ABSTRACT

Since hypoxia might contribute to the development of muscle atrophy, we wished to provide direct evidence linking hypoxia to muscle atrophy. By evaluating protein degradation and synthesis in hypoxic myotubes we found a significant reduction in total protein content. Using functional assays we observed protein degradation elevation in the first 24 h while synthesis was maintained during this period and then significantly decrease at 48 h. These results demonstrate a temporal regulation of protein homeostasis, whereby elevated protein degradation is followed by a reduction in synthesis. These results are comparable to the cellular adaptation seen during development of muscle atrophy.
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1. Introduction

Muscle atrophy is a common clinical manifestation occurring in a variety of hypoxemic conditions, including chronic obstructive pulmonary disease (COPD) [1–3]. In these subjects, peripheral muscle wasting decreases functional capacity and increases mortality [4]. We and others have hypothesized that hypoxemia and the resulting tissue hypoxia is among the factors that might contribute for the development of muscle atrophy [3,5]. However, this statement has not been directly addressed.

Muscle mass maintenance relies on a fine regulation between contractile protein degradation and synthesis. In wasting conditions, contractile protein degradation is mostly achieved through the activation of the ubiquitin-proteasome (Ub-P'some) [6]. Since this system is unable to directly break up the actomyosin structure found in myofibrils [7], a preliminary rate-limiting step is required to fragment actomyosin into substrates that will be degraded by the Ub-P'some. Among these substrates, actin fragments represent a reliable marker for contractile protein degradation [8].

The major cascade involved in protein synthesis is the insulin-like growth factor-1 (IGF-1)/phosphatidylinositol-3 kinase (PI3K)/Akt pathway. Akt is a kinase which, upon phosphorylation, ensures cell survival, differentiation and protein synthesis by modulating the activity of numerous targets including glycogen synthase kinase-3 (GSK-3) [9].

In this study, we aimed to provide direct evidences demonstrating that hypoxia is involved in the instigation of cellular events compatible with the initiation and the development of muscle atrophy. We hypothesized that hypoxic exposure of L6 myotubes would result in an elevation of contractile protein degradation and the activation of the Ub-P'some system with a concomitant reduction in protein synthesis and activity of the IGF-1/PI3K/Akt pathway.

2. Materials and methods

2.1. Cell culture and hypoxic exposures

L6 rat myoblasts (ATCC, Manassas, VA, USA) were grown in Dulbecco's modified Eagle's medium (DMEM) with 1% glucose (HyClone, Logan, UT, USA), 10% fetal bovine serum, penicillin 50 U/ml, and streptomycin 50 μg/ml (HyClone). Confluent myoblasts (80%) were placed in differentiation medium made of DMEM with 1% glucose, 2% horse serum, penicillin 50 U/ml, and streptomycin 50 μg/ml. After 4 days, differentiated myotubes were transferred to a chamber maintained at 1% O_2, 5% CO_2, 94% N_2 (ProOx system, BioSpherix, Redfield, NY, USA). Prior to hypoxic exposure, differentiation medium was replaced with medium preconditioned overnight in 1% O_2 atmosphere. After designated exposures, hypoxic cells were lysed in 1% O_2 atmosphere inside a C-Shuttle Glovebox (Biospherix) according to the subsequent protocols.

Abbreviations: COPD, chronic obstructive pulmonary disease; Ub-P'some, ubiquitin-proteasome; DMEM, Dulbecco's modified Eagle's medium; E3, ubiquitin ligase; EDTA, ethylenediamine tetraacetic acid; GSK-3, glycogen synthase kinase-3; IGF-1, insulin-like growth factor-1; PBS, phosphate buffered saline; PI3K, phosphatidylinositol-3 kinase

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2.2. Cell viability assessments

Cell stress induced by hypoxia was assessed by measuring lactate dehydrogenase (LDH) leakage [10] using LDH-Cytotoxicity Assay Kit (BioVision, Mountain View, CA, USA) according to the manufacturer's instructions. For each experimental condition, percentage of cytotoxicity was calculated using the following formula: (LDH media/(LDH media + LDH cells)) × 100. General appearance of myotubes was examined using a Nikon Eclipse TE300 inverted microscope (Nikon Corporation, Tokyo, Japan) and images were captured with a Nikon 950 digital camera (Nikon Corporation). Number of nucleus was assessed using 4′,6-diamidino-2-phenylindole (DAPI) staining (Invitrogen, Carlsbad, CA, USA). Briefly, myotubes were grown on four wells Lab-Tek™ chamber slides (Thermo Fisher Scientific, Waltham, MA, USA). After designated expositions, cells were fixed with 2% paraformaldehyde for 15 min and DAPI was added for 2 min at a final concentration of 30 nM in phosphate buffered saline (PBS) 1X supplemented with 1% bovine serum albumin. Images were captured using a Nikon Eclipse E600 microscope (Nikon Corporation) and all nuclei from six random separate fields were counted.

2.3. Actomyosin breakdown measurements

The 14-kDa actin fragment accumulation, an indicator of actomyosin degradation, was measured as previously described [8]. Cells were scrapped in a hypotonic lysis buffer [5 mM Tris–HCl pH 8.0, 1 mM β-mercaptoethanol, 1% glycerol, 1 mM ethylenediamine tetraacetic acid (EDTA), 1 mM EGTA, protease inhibitor cocktail set III (EMD biosciences, San Diego, CA, USA)]. After centrifugation (13 000 rpm, 4 °C, 10 min), supernatant was collected for detection of accumulated 14-kDa actin fragment by Western blot using an anti-actin antibody which recognizes the carboxy-terminal 11 amino acids (1:500, #A2066; Sigma–Aldrich, St. Louis, MO, USA). Anti-actin antibody was then detected using a secondary antibody coupled with horseradish peroxidase (1:5000, #7074; Cell Signaling Technology Inc.). Results were normalized to tubulin (1:20 000; Sigma–Aldrich).

2.4. Proteasome activity assays

Proteasome activity assays were performed using a modified protocol [11]. Briefly, cells were washed in ice-cold PBS and scrapped in lysis buffer [50 mM Tris–HCl pH 7.5, 5 mM $MgCl_2$, 250 mM sucrose, 1 mM DTT, 200 μM PMSF, protease inhibitor cocktail set III (EMD biosciences)]. Lysate was grinded on ice and centrifuged (10 000×g, 4 °C, 20 min). Supernatant was collected and centrifuged (100 000×g, 4 °C, 45 min). Resulting pellet was resuspended [50 mM Tris–HCl pH 7.5, 5 mM $MgCl_2$, 20% glycerol] and 10 μg of protein was added to 50 μl of reaction buffer [50 mM Tris–HCl pH 8, 10 mM $MgCl_2$, 2U Apyrase (Sigma–Aldrich)] containing either 100 μM Suc-LLVY-AMC or 600 μM Z-LLE-AMC (Sigma–Aldrich) to quantify chymotrypsin-like and caspase-like proteasomal activities, respectively. After 15 min of incubation at 37 °C, fluorescence was measured (excitation 360 nm, emission 460 nm) (Fluoroskan Ascent; Labsystems, Helsinki, Finland).

2.5. Real-time PCR

Total RNA was isolated using TRIzol® Reagent (Invitrogen) and its quantity and purity were determined by spectrophotometry. Reverse transcription was performed using Quantitect™ Reverse Transcription Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instruction. Real-time PCR was performed with the Quantitect™ SYBR® Green PCR Kit (Qiagen) in an Opticon® 2 (MJ Research, Waltham, MA, USA). 18S mRNA was used as an internal control. Primers used were (sense and antisense, 5′–3′): Atrogin-1 gtccagagagtcggcaagtc, gtcggtgatcgtgagacctt (141pb); MuRF1 tgaccaaggaaaacagccaccag, tcactcttcttctcgtccaggatgg (88pb) [12]; 18S acggaagggcaccaccagga, caccaccacccacggaatcg (127pb) [13]. Real-time PCR were analyzed using the $2^{-\Delta\Delta C_T}$ method [14].

2.6. Protein synthesis measurements

Total protein synthesis was appraised by measuring cellular incorporation of L-[2,3,4,5,6-^{3}H]Phenylalanine (Amersham, Buckinghamshire, UK). Myotubes grown in a six wells plate were incubated in differentiation medium containing L-[2,3,4,5,6-^{3}H]-Phenylalanine (0.5 μCi/ml). Controls with the proteasome inhibitor epoxomicin (10 nM) were used to inhibit proteolysis. In a subsequent step, cells were washed in ice-cold PBS and incubated overnight in a lysis buffer [PBS + 1% SDS] at 4 °C. L-[2,3,4,5,6-^{3}H]-Phenylalanine uptake was measured in the lysates using a Tri-Carb® 2100TR liquid scintillation counter (Perkin–Elmer Life Sciences, Boston, MA, USA).

2.7. Western blotting

Myotubes were washed with ice-cold PBS and incubated on ice in lysis buffer [10 mM Tris–HCl pH 7.4, 150 mM NaCl, 2 mM EDTA, 2% Triton X-100, protease inhibitor cocktail set III (EMD biosciences), phosphatase inhibitor cocktail 1 and 2 (Sigma–Aldrich)] for 30 min. Western blots were performed with 60 μg of whole cell extracts using standard SDS–PAGE procedures. After transfer on a nitrocellulose membrane, immunoblotting with anti-Akt (1:2000), anti-phospho-Akt (Ser473) (1:1000), anti-GSK-3β (1:1000) or anti-phospho-GSK-3β (1:1000) (Cell Signaling Technology, Danvers, MA, USA) was performed. Proteins of interest were detected using a secondary antibody coupled with horseradish peroxidase (1:5000, #7074; Cell Signaling Technology Inc.). Results were normalized to tubulin (1:20 000; Sigma–Aldrich).

2.8. Akt kinase activity assays

Akt kinase activity was evaluated using an Akt Activity Immunoassay Kit (EMD biosciences) according to the manufacturer's instructions.

2.9. Statistical analyses

All statistical analyses were performed using JMP® 7.0 (SAS Institute, Cary, NC, USA). Comparisons of experimental conditions were carried out by ANOVA. P-value <0.05 was considered significant.

3. Results

3.1. Cell viability is minimally affected in hypoxic myotubes

We first evaluated the hypoxic stress on myotubes by measuring LDH leakage in the culture media as an indicator of membrane integrity. In hypoxic myotubes, we found a significant increase in LDH leakage (31.5 ± 3.4%, $n = 3$; $P < 0.05$) after 48 h of exposure when compared to matching normoxic controls (Fig. 1A). Insignificant change was observed at 24 h (2.5 ± 1.6%, $n = 3$; $P > 0.05$). Despite an increase in LDH leakage, the main cellular architecture of myotubes is preserved even if minor to moderate structural changes are observed after 48 h of hypoxic exposure (Fig. 1B). Finally, nuclei number present at 48 h in hypoxic myotubes (Fig. 1C) was decreased by 11.3% ($n = 6$, $P < 0.05$). Overall, these results indicate that our hypoxic protocol has a sig-

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