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Post-transcriptional regulation of autophagy in C2C12 myotubes following starvation and nutrient restoration

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ABSTRACT

In skeletal muscle, autophagy is activated in multiple physiological and pathological conditions, notably through the transcriptional regulation of autophagy-related genes by FoxO3. However, recent evidence suggests that autophagy could also be regulated by post-transcriptional mechanisms. The purpose of the study was therefore to determine the temporal regulation of transcriptional and post-transcriptional events involved in the control of autophagy during starvation (4 h) and nutrient restoration (4 h) in C2C12 myotubes. Starvation was associated with an activation of autophagy (decrease in mTOR activity, increase in AMPK activity and Ulk1 phosphorylation on Ser467), an increase in autophagy flux (increased LC3B-II/LC3B-I ratio, LC3B-II level and LC3B-positive punctate), and an increase in the content of autophagy-related proteins (Ulk1, Atg13, Vps34, and Atg5–Atg12 conjugate). Our data also indicated that the content of autophagy-related proteins was essentially maintained when nutrient sufficiency was restored. By contrast, mRNA level of *Ulk1*, *Atg5*, *Bnip3*, *LC3B* and *Gabarapl1* did not increase in response to starvation. Accordingly, binding of FoxO3 transcription factor on LC3B promoter was only increased at the end of the starvation period, whereas mRNA levels of *Atrogin1/MAFbx* and *MuRF1*, two transcriptional targets of FoxO involved in ubiquitin–proteasome pathway, were markedly increased at this time. Together, these data provide evidence that target genes of FoxO3 are differentially regulated during starvation and that starvation of C2C12 myotubes is associated with a post-transcriptional regulation of autophagy.

1. Introduction

Gain or loss of skeletal muscle mass is a dynamic process largely regulated by the rate of protein synthesis and protein degradation. During many chronic diseases (cancer, sepsis, acquired immunodeficiency syndrome, cardiac and renal failures), the rate of protein degradation becomes higher than the rate of protein synthesis leading to an altered nitrogen balance, and ultimately to a loss of skeletal muscle mass and muscle wasting (Gordon et al., 2013; Sandri, 2013).

Accelerated protein breakdown has been largely attributed to the activation of ubiquitin–proteasome system (Bodine et al., 2001; Gomes et al., 2012; Lecker et al., 2006). Increase in ubiquitin–proteasome system strongly depends on the transcriptional activation of the muscle-specific E3-ubiquitin ligase, Atrogin1/muscle atrophy F-box (MAFbx) (Bodine et al., 2001;

Abbreviations: ACC, acetyl-CoA carboxylase; Akt/PKB, protein kinase B; AMPK, AMP-activated protein kinase; ANOVA, analysis of variance; Atg, autophagy-related gene; Bnip3, B-cell lymphoma 2 interacting protein 3; DMEM, Dulbecco's modified Eagle medium; DPBS, Dulbecco's phosphate-buffered saline; FIP200, FAK family kinase-interacting protein of 200 kDa; E3-ubiquitin ligase, enzyme type 3 ubiquitin ligase; FOXO, forkhead box protein O; LC3B, microtubule-associated protein light chain 3; MAFbx, muscle atrophy F-box; mTOR, mammalian target of rapamycin; mTORC1, mammalian target of rapamycin complex 1; MuRF1/Trim63, muscle ring finger-1; P0, Rplp0; p70S6K, ribosomal protein S6 kinase; P/S, penicillin–streptomycin; rpS6, ribosomal protein S6; SEM, standard error of the mean; Ser, Serine; Thr, Threnonine; Ppia, cyclophilin-A; Ulk1, unc-51 like autophagy activating kinase 1; vps34, vacuolar protein sorting 34.

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Gomes et al., 2012) and muscle ring finger-1 (MuRF1) (Bodine et al., 2001). Conversely, deficiency in either *Atrogin1*/*MAFbx* or *MuRF1* partially prevents muscle atrophy (Bodine et al., 2001). An important mechanism for the transcriptional regulation of *Atrogin1/MAFbx* and *MuRF1* involves the inhibition of Akt and the subsequent activation and nuclear translocation of Forkhead box protein O (FoxO) family of transcription factors (Sandri et al., 2004; Stitt et al., 2004).

Autophagy–lysosome system is also an important proteolytic system in regulating the turnover of muscle fiber components both in basal and catabolic conditions (Bonaldo and Sandri, 2013). During macroautophagy, a portion of cytoplasm is enclosed by an isolation membrane called phagophore to form an autophagosome, which then fuses with lysosomes to form autolysosomes where the sequestrated internal material is finally degraded (Mizushima, 2007; Sandri, 2010). Proteins encoded by autophagy-related genes are essential mediators of autophagy by controlling membrane commitment, membrane growth and fusion to form an autophagosome (Bonaldo and Sandri, 2013). FoxO3 transcription factor is necessary for the induction of autophagy *in vivo*. In skeletal muscle or myotubes undergoing atrophy, the activity of the Akt/mammalian target of rapamycin (mTOR) pathway decreases, leading to the nuclear translocation and activation of FoxO3, and the transactivation of its target genes including *microtubule-associated protein light chain 3* (*LC3*), *Beclin 1*, *B-cell lymphoma 2 interacting protein 3* (*Bnip3*), *Gabarapl1 or autophagy-related gene 12* (*Atg12*) (Mammucari et al., 2007; Sacheck et al., 2004; Zhao et al., 2007). The transcriptional control of autophagy-related gene expression is therefore an important regulatory step during catabolic conditions (Lecker et al., 2004; Mammucari et al., 2007; Zhao et al., 2007).

However, a transcriptional response is not systematically observed when autophagy is activated. It has thus been reported that activation of autophagy in response to amino acid deprivation in HeLa cells was not associated with an increase in the mRNA level of multiple autophagy-related genes (Khambu et al., 2011). Furthermore, an important post-transcriptional regulation of autophagy has also recently been described. Ulk1, which is necessary for autophagy induction, is phosphorylated and activated by AMP-activated protein kinase (AMPK) upon starvation (Egan et al., 2011; Ganley et al., 2009; Hosokawa et al., 2009; Jung et al., 2009; Kim et al., 2011). By contrast, nutrient sufficiency increases mTOR activity and prevents Ulk1 activation by disrupting the interaction between Ulk1 and AMPK (Kim et al., 2011). Altogether, these data suggest that post-transcriptional mechanisms could also be important in the control of autophagy.

The intent of the present investigation was therefore to determine the temporal regulation of transcriptional and post-transcriptional events involved in the control of autophagy during starvation (4 h) and nutrient restoration (4 h) in C2C12 myotubes.

2. Materials and methods

2.1. Cell culture

C2C12 (ATCC, CRL-1772) were cultured in Dulbecco's modified Eagle's medium (DMEM) (PAA, E15-843) supplemented with 10% fetal bovine serum (PAA, A15-151) and 1% penicillin–streptomycin (P/S) (PAA, Sigma P-0781) at 37 °C and 5% CO_2 in air. At 75% confluence, cells were allowed to differentiate for 6 days in DMEM supplemented with 2% horse serum (HS) (PAA, B11-021) and 1% P/S. Myotubes were then submitted to a metabolic challenge consisting in 4 h of starvation followed by 4 h of nutrient restoration: C2C12 myotubes were switched to a glucose-free and amino acid-free medium (Dulbecco's phosphate buffer saline (DPBS)) (PAA, H15-001) containing 2% HS and 1% P/S. Control myotubes were cultured in DMEM containing 2% HS and 1% P/S. After 15, 30, 60, 120 and 240 min of starvation, myotubes were washed and harvested. C2C12 myotubes treated with DPBS for 4 h were also harvested 15, 30, 60, 120 and 240 min after restoration of nutrient sufficiency (DMEM supplemented with 2% HS and 1% P/S).

2.2. Time-lapse image analysis

Six day-differentiated C2C12 myotubes in 6-well culture plates were treated with DPBS containing 2% HS and 1% P/S. After 4 h, myotubes were replenished with DMEM containing 2% HS and 1% P/S. Image acquisition was performed with a Zeiss Cell Observer Z1 microscope equipped with a humidified air/CO_2 and temperature controlled chamber. Image acquisition was recorded with a 10× objective every 5 min during 8 h. Myotube area was quantified from six different fields by using Axion Vision 4.7 software. The area quantification was realized by measuring the area of 10 different myotubes at the indicated time points.

2.3. Immunofluorescence staining

After differentiation on coverslips for 6 days and subjected to starvation for 0, 60 and 240 min, cultured cells were fixed with 4% paraformaldehyde for 10 min at room temperature, permeabilized with Triton 0.1% for 2 min at room temperature. Cells were then blocked for 1 h at room temperature with PBS containing BSA 1%, chicken serum 5% and Tween 0.1%, and incubated overnight at 4 °C with anti-LC3b antibody (Cell Signaling, USA, 2775) in blocking solution. After washing in PBS, cells were incubated with Alexa Fluor 488 anti-rabbit antibody (Life Technologies, USA) for 1 h at room temperature. The nuclei were counterstained with Hoechst 33342 for 10 min at room temperature. After mounting in fluorescence mounting medium, the cells were imaged using IX81 Olympus microscope (Olympus America, PA) equipped with Lambda DG-4 (Sutter Instruments, Novato, CA) wide field illumination system and Evolve 512 EMCCD (Photometrics, Tucson, AZ) camera using Slidebook 5.0 software (Intelligent Imaging Innovations, Inc., Denver, CO).

2.4. Protein isolation

Myotubes harvested at the indicated time points during starvation and nutrient restoration were rinsed and scraped with 200 μl of buffer consisting in 50 mM Tris (pH 7.4), 100 mM NaCl, 2 mM EDTA, 2 mM EGTA, 50 mM β-glycerophosphate, 50 mM NaF, 1 mM sodium orthovanadate, 120 nM okadaic acid and 1% Triton X-100. Cells were then submitted to three consecutive cycles of freeze–thawing in liquid nitrogen and sonication (10 s on ice). Myotubes were then centrifuged at 10,000 × *g* for 10 min at 4 °C. Protein concentration of the supernatant was spectrophotometrically measured at 750 nm using Bio-Rad protein assay (Bio-Rad).

2.5. Luminex analysis

Fluorescent capturing beads coupled to antibodies directed against Akt Ser473 (Millipore, 46-677) and p70S6K Thr412 (Millipore, 46-629) were incubated for 2 h with 50 μl of protein fractions (1:10). 96-well plates were then washed, incubated with 25 μl of biotinylated antibodies for 30 min, followed by the incubation with a streptavidin–phycoerythrin solution for 10 min. The analysis consisted of a double-laser fluorescence detection, which allowed simultaneous identification of the target protein through the red fluorescence emission signal of the bead and quantification of the target protein through the fluorescence intensity of phycoerythrin. Fluorescence intensities were recorded on a Bio-Plex™ 200 System