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AMP-activated protein kinase stabilizes FOXO3 in primary myotubes

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ABSTRACT

AMP-activated protein kinase (AMPK) is a critical enzyme in conditions of cellular energy deficit such as exercise, hypoxia or nutritional stress. AMPK is well known to regulate protein degradation pathways notably through FOXO-related axis. In this study, we investigated the implication of AMPK activation in FOXO3 expression and stability in skeletal muscle primary myotubes. First, time course and dose response studies revealed optimal AICAR treatment duration and dose in skeletal muscle cells. Then, experiments with cycloheximide treatment of primary myotubes highlighted that AICAR infusion extends FOXO3 protein half-life. Our results also showed that AICAR treatment or nutrient depletion increases FOXO3 expression in primary myotubes and the expression of the mitochondrial E3 ligase Mul1 involved in mitochondrial turnover (mitophagy). In AMPK KO cells, nutrient depletion failed to alter the level of some FOXO3-dependent atrophic genes, including LC3B, BNIP3, and the mitochondrial E3 ligase Mul1, but not the expression of other genes (*i.e.* FOXO1, Gabarapl1, MAFbx, MuRF1). In summary, our data highlight that AMPK stabilizes FOXO3 and suggest a role in the first initiation step of mitochondrial segregation in muscle cells.

1. Introduction

AMP-activated protein kinase (AMPK) is a serine-threonine kinase considered as a key enzyme in conditions of cellular energy deficit. AMPK is a heterotrimeric complex composed of a catalytic subunit (AMPK-α) and two regulatory subunits (AMPK-β and AMPK-γ) [1]. AMPK is activated by a large variety of stresses including hypoxia [2], exercise [3–5], food deprivation [6], and drugs such as AICAR [7]. In response to energy stress, AMPK promotes metabolic changes to maintain cell survival [1]. In skeletal muscle, AMPK activation results in the inhibition of protein synthesis, cell growth and hypertrophy [8]. Thus, AMPK reduces both the initiation and the elongation of ribosomal peptide synthesis [9,10] by inhibition of the mammalian/mechanistic target of rapamycin complex 1 (MTORC1) pathway [11,12]. In the last decade, several works associated the activation of AMPK with stimulation of myofibrillar proteolysis in muscle cells [13,14].

Protein degradation is mediated by two canonical conserved pathways: the ATP-dependent ubiquitin-proteasome and the autophagy-lysosomal systems. The first one involves a cascade of enzymatic reactions that label substrate proteins with ubiquitin chains for degradation by the 26S proteasome. This system involves the activity of an E3 ubiquitin ligase that confers substrate specificity for ubiquitination. Two majors E3 ligases have been initially described to be essential for muscle proteolysis, MuRF1 (Muscle RING Finger 1), and atrogin-1/MAFbx (Muscle Atrophy F-box) [15,16], but recently, the role of the mitochondrial E3-ligase Mul1 has been assessed in mitochondrial turnover and dynamics, and apoptosis [17–19]. The second pathway, the autophagy-lysosomal system, is a highly-conserved eukaryotic stress and survival response notably involved in organelle turnover. Several stages allow incorporation of long-lived cytosolic proteins and organelles (*i.e.* mitochondria, ribosome, peroxisomes) into a double-membrane vesicule (autophagosome) which is then degraded by lysosomal hydrolases into a lysosome [37]. Autophagy requires transcription of autophagy-specific genes (Atgs) for the formation of autophagosomes [20] and implies specifically two ubiquitin-like conjugation systems for the elongation and maturation of autophagosomal membranes (*i.e.* the Atg12/Atg5/Atg16 complex, and Atg8, also known as microtubule-associated protein 1 light chain 3

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(LC3) in mammals). Of note, two other complexes are important in autophagy process, the unc-51 like kinase (Ulk1)/Atg1 and the Beclin1/vacuole protein sorting 34 (Vps34)/PI3K complexes, for initiation of autophagosome formation and nucleation of autophagosomal membranes, respectively [21].

During skeletal muscle wasting, including starvation-induced atrophy, the Forkhead box class O family member protein 3 (FOXO3) regulates the transcription of several E3 ligases (*i.e.* MAFbx, MuRF1 and potentially Mul1) and Atgs [22–24,38]. Recent data also showed that Mul1 is involved in the turnover of mitochondrial proteins and consequently has a role in the control of the quality of mitochondrial population [17,19]. FOXO proteins are highly conserved transcription factors with important roles in cellular homeostasis. Four FOXO members are expressed in humans (*i.e.* FOXO1, FOXO3, FOXO4, and FOXO6), but the first three members are the most studied in skeletal muscle cells. Few of post-translational modifications of FOXO proteins have been investigated in skeletal muscle [25]. However, it was found that FOXO3 activity is negatively regulated by acetylation of lysine 262 and ubiquitination in muscle cells. Thus, the histone acetyl-transferase p300 targets FOXO3, leading to its cytosolic relocalization and proteasomal degradation through the E3 ligase murine double minute 2 (Mdm2) [26]. FOXO proteins are also regulated by phosphorylation through the IGF-1/PI3K/Akt signaling pathway and AMPK [27–30]. However, to the best of our knowledge, no information is yet available regarding the implication of AMPK in FOXO3 stability in muscle cells. Some data strongly suggest that FOXO3 nuclear relocalization is not necessarily required to increase its transcriptional function and phosphorylation by AMPK may also control FOXO3 protein stability [27,30].

In the present work, we addressed the question of the potential role of AMPK in FOXO3 stability and examinated the regulation of FOXO3-dependant mitophagic-related pathways by AMPK.

2. Materials and methods

2.1. Reagents

The AMP adenosine analog AICAR and cycloheximide (CHX) were purchased from Sigma.

2.2. Cell cultures and AMPKα (−/−) primary cells

Primary cultures were prepared from male mice from our own breeding stocks. All animals were treated in accordance with institutional and national guidelines. Briefly, mice satellite cells were isolated from the whole muscles of the paws. Cells were plated at a density of approximatively 2×10^4 cell/cm^2 on Matrigel-coated Petri dishes (BD Biosciences) in 80% Ham's-F10 medium containing glutamine, penicillin, and amphotericin B (Invitrogen), supplemented with 20% horse serum. After 2 days, cells were washed with Ham's-F10 and placed in complete medium supplemented with 5 ng/ml basic fibroblast growth factor. Differentiation was induced in subconfluent cells by removing the basic fibroblast growth factor.

AMPKα double KO (α1, α2) cells were obtained from Dr B. Viollet (Institut Cochin, Paris) [31,32].

2.3. Starvation

Cells were washed three times with PBS and incubated with 1 ml modified PBS (100 mM NaCl, 5 mM KCl, 1.5 mM MgSO4, 50 mMNaHCO3, 1 mMNaH2PO4, 2 mMCaCl2) without serum at 37 °C during the indicated times.

2.4. Western immunoblotting

Cells were washed twice and homogenized in lysis buffer [50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EGTA, 100 mM NaF, 5 mM Na3VO4, 1% Triton X-100, and protease inhibitor mixture (P8340, Sigma-Aldrich)]. Cellular debris were removed by centrifugation at 10,000 g for 10 min (4 °C). Protein concentrations were measured using a Pierce BCA protein assay kit (23225, Thermo Scientific). Proteins (70 μg) were denatured and loaded onto 8 and 15% SDS-polyacrylamide gels before electrophoretic transfer onto a nitrocellulose membrane (Bio-Rad). After transfer, membranes were blocked with 50 mM Tris-HCl pH 7.5, 150 mM NaCl, and 0.1% Tween 20 (TBS-T) containing 5% skimmed milk or BSA and incubated overnight at 4 °C with primary antibodies. Membranes were washed three times for 10 min with TBS-T and incubated for 1 h with a peroxidase conjugated secondary antibody (Cell signaling). Membranes were washed again three times for 5 min and immunoblots were revealed by using a Pierce ECL kit (32106, Thermo Scientific) according to the manufacturer's instructions.

2.5. Antibodies

Anti- Phospho-AMPK (Thr172), total AMPK and FOXO3 were purchased from Cell Signaling, anti- Mul1 was obtained from Abcam, anti- MFN2 from Santa Cruz, anti- α-tubulin from Sigma-Aldrich.

2.6. Reverse transcription/polymerase chain reaction

Total RNA was prepared by using RNeasy (Qiagen), according to the manufacturer's instructions. Genomic DNA from all samples was removed with the DNA-free DNase digestion kit (Ambion) according to the manufacturer's protocol. First-strand cDNA was synthesized from 1 μg total RNA by AMV reverse transcriptase (Promega) in the presence of 500 ng oligo dT15 primer. A negative control without reverse transcriptase was included in each cDNA synthesis. The polymerase chain reaction (PCR) was performed for 26 and 32 cycles, depending on the primer sets used. An internal standard (expression of D-glyceraldehyde-3-phosphate dehydrogenase; GAPDH) for every PCR was included and data were normalized to the internal standard. PCR was performed in GoTaq buffer containing 1.5 mM MgCl2 (Promega), 0.25 μl GoTaq, 200 μM each dNTP and 0.5 μM appropriate sense and antisense primers in a reaction volume of 50 μl. Amplification was carried out by using the following protocol: 150 s initial denaturation at 95 °C, 45 s denaturation at 95 °C, 45 s annealing at 60 °C and 45 s extension at 72 °C. Products were run on a 1.5–1.8% (w/v) agarose gel, stained with 15 μg/ml ethidium bromide and visualized under UV illumination. Band intensity was measured from six independent experiments in duplicate by using ImageJ software and normalized to GAPDH signal. Primers used for reverse transcription/PCR (RT-PCR) analysis were:

BNIP3-F: CCTGTCGCAGTTGGGTTC, BNIP3-R: GAAGTGCAGTTCTACCCAGGAG, FOXO3-F: GGAAATGGGCAAAGCAGA, FOXO3-R: AAACGGATCACTGTCCACTTG, Mul1-F AGGGCATTCTTTCAGAAGCA, Mul1-R GGGGTGGAACTTCTCGTACA, RPS9-F CGGCCCGGGAGCTGTTGACG, RPS9-R CTGCTTGCGGACCCTAATGT, MAP11C3b_(LC3)-F CCCCACCAAGATCCCAGT, MAP11C3b_(LC3)-R CGCTCATGTTCACGTGG.

2.7. Statistics

Western blot densitometry analysis of immunoblots from minimum three independent experiments was performed using Image J software. The statistical analyses were performed using GraphPad

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