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AMP-activated protein kinase stimulates myostatin expression in C2C12 cells

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ABSTRACT

AMP-activated protein kinase (AMPK) is a master regulator of energy metabolism in skeletal muscle; AMPK induces muscle protein degradation but the underlying mechanisms are unclear. Myostatin is a powerful negative regulator of skeletal muscle mass and growth in mammalian species. We hypothesized that AMPK stimulates myostatin expression, which provides an explanation for the negative role of AMPK in muscle growth. The objective of this study is to demonstrate that AMPK stimulates myostatin expression using C2C12 cells as a model. Activation of AMPK by 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) dramatically increased the mRNA expression and protein content of myostatin in C2C12 myotubes, and to a lesser degree in myoblasts. Metformin, another AMPK activator, also stimulated myostatin expression at low concentrations. In addition, ectopic expression of AMPK wild-type α subunit (enhancing AMPK activity) and AMPK K45R mutant (knockdown AMPK activity) enhanced and reduced myostatin expression, respectively. These results indicate that AMPK stimulates myostatin expression in C2C12 cells, providing an explanation for the negative effect of AMPK on muscle growth.

1. Introduction

AMP-activated protein kinase (AMPK) is a well-characterized sensor of cellular energy status and metabolic stress [1]. Changes in the cellular energy state activate AMPK through several mechanisms involving allosteric regulation of AMPK, activation by upstream AMPK kinase, and diminished activity of phosphatases [2]. AMPK is switched on by an increase in the AMP/ATP ratio, leading to the phosphorylation of AMPK at Thr 172 by AMPK kinases [3]. Once activated, AMPK promotes glucose and fatty acid uptake and oxidation, while inhibiting lipid synthesis in cells [4] and repressing energy consuming processes e.g. generation of fatty acids [5] and protein synthesis [6].

Regulation of muscle mass depends on a fine balance between muscle protein synthesis and degradation. Activation of AMPK in skeletal muscle down-regulates protein synthesis via inhibition of mammalian target of rapamycin (mTOR) kinase, a key mediator of muscle protein synthesis [7]. It has been reported recently that AMPK activation inhibits cell cycle transition, reducing differentiation of myoblasts into myotubes, and induces muscle protein degradation [8–11]. We previously demonstrated that AMPK enhances muscle protein degradation through promoting the expression of two muscle-specific ubiquitin ligases, muscle atrophy F box (MAFbx) and muscle RING finger 1 (MuRF1) [10]. However, the exact mechanisms linking AMPK to enhanced expression of ubiquitin ligases and enhanced muscle protein degradation remains unclear.

Myostatin is a growth and differentiation factor (GDF 8) belonging to the transforming growth factor (TGF)-β superfamily, which acts as a negative regulator of muscle mass [12]. The deletion of myostatin in mice causes a dramatic and widespread increase in skeletal muscle mass, resulted from both hyperplasia and hypertrophy of muscle fibers [12–14]. Myostatin is capable of inducing muscle atrophy via its inhibition of myoblast proliferation, increasing ubiquitin-proteasomal activity and down-regulating the mTOR protein synthesis pathway [15]. Increases in myostatin mRNA expression induced by both burn injury and dexamethasone injection in rats correlates with the loss of muscle mass [16–18]. With the above findings, we speculate that AMPK stimulates myostatin expression, providing an important novel mechanism linking AMPK to muscle protein degradation and muscular atrophy. Our data demonstrate that AMPK promotes myostatin expression in C2C12 myotubes.

2. Materials and methods

2.1. Chemicals and antibodies

5-Aminoimidazole-4-carboxamide-1-β-D-ribonucleoside (AICAR), a specific activator of AMPK [19], and all other chemicals

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were purchased from Sigma Aldrich (St. Louis, MO). Antibodies against AMPKα, phospho-AMPK at Thr 172, and horseradish peroxidase linked secondary antibody were purchased from Cell Signaling (Danvers, MA). GDF8 (#sc-6885-R) antibody was from Santa Cruze Biotechology, Inc. Anti-ß-tubulin antibody was obtained from Developmental Studies Hybridoma Bank (DSHB, Iowa City, IA).

2.2. C2C12 cell culture

C2C12 myoblasts were obtained from the American Type Culture Collection (ATCC; Manassas. VA) and grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics in a humidified atmosphere of 95% air and 5% CO at 37 °C. At 90% confluence, C2C12 myoblasts in 6-well plates were incubated with AICR (0, 0.25, 0.5 and 1.0 mM). At confluence, myoblasts were induced to fuse into myotubes by using DMEM containing 2% horse serum (differentiation medium) for 3 d. Then, C2C12 myotubes in 6-well plates were incubated with AICAR (0, 0.25, 0.5 and 1.0 mM) or metformin (0, 0.5, 1.5 and 2.0 mM) for 24 h in the differentiation medium.

2.3. Real-time quantitative PCR (RT-PCR)

Total mRNA was extracted from muscle using TRI reagent (Sigma, St. Louis, MO) and reverse transcribed into cDNA using an iScriptTM cDNA synthesis kit (Bio-Rad, Hercules, CA). RT-PCR was performed on a CFX connected TM Real-Time PCR detection system (Bio-Rad) using SYBR Green RT-PCR kit from Bio-RAD. PCR conditions were as follows: 20 s at 95 °C, 20 s at 56 °C, and 20 s at 72 °C for 35 cycles. Primer sequence was as following: myostatin forward (5′-AATCCCGGTGCTGCCGCTAC-3′) and reverse (5′-GTCGGAGTGCAGCAAGGGCC-3′); 18S rRNA forward (5′-TGCTGTCCCTGTATGCCTCT-3′) and reverse (5′-TGTAGCCACGCTCGGTCA-3′). After amplification, a melting curve (0.01 C/s) was used to confirm product purity, and the PCR products were electrophoresed to confirm the targeted sizes. Results are expressed relative to 18S.

2.4. Lentivirus transduction of C2C12 cells

Plasmids AMPK WT (Plasmid 15991) and AMPK K45R (Plasmid 15992) were obtained from Addgene (Cambridge, MA) [20,21], and sub-cloned into a lentiviral vector (Plasmid 17445) using PCR with primers: Forward (5′-GTGTaCcGGtATTCGCCATGGAGCAGAA-3′) and reverse (5′-CCGGgtcgacGTTATCAACGGGCTAAAGCAGTGAT-3′). As a result, the amplicons had two new restriction sites, Age1 and Sall. These newly constructed vectors were packaged into vesicular stomatitis virus glycoprotein pseudotyped lentiviral vector virions as previously described [22] and used for transduction. Briefly, C2C12 cells were seeded on 12 plates to reach 90% confluence and were tranduced with AMPK WT and AMPK K45R lentiviral constructs. Transduction efficiency was monitored by the transduction of a control eGFP lentiviral vector virions. Cells were incubated at 37 °C in 5% CO_2. After 24 h, protein was harvested for western blot analysis.

2.5. Immunoblotting analysis

Western blotting was conducted as previously described [23] using an Odyssey Infrared Imaging System (Li-COR Biosciences, Lincoln, NE). Density of bands was quantified and then normalized according to the tubulin content [20].

2.6. Statistical analysis

Data were analyzed by GLM procedure (SAS Inst., Inc., Cary, NC). Statistical significances were determined by the Tukey's Studentized Range test. $P < 0.05$ was considered significant.

3. Results

3.1. AICAR enhanced myostatin expression in C2C12 myotubes

C2C12 myotubes were treated with various doses of AICAR for 24 h. AICAR treatment induced dose-dependent phosphorylation of AMPK (Fig. 1A), showing the activation of AMPK in response to AICAR. Consistently, the myostatin protein content was increased in a dose dependent manner (Fig. 1B). Such increase in myostatin protein content was at least partially due to elevated mRNA expression (Fig. 1C). Interestingly, the expression of myostatin mRNA in response to AICAR treatment was much more robust in myotubes that myoblasts (Fig. 1D).

To further test the effect of AMPK on myostatin expression, we used another chemical, metformin, which activates AMPK through a different mechanism. We used different concentration of metformin (0, 0.5, 1.5 and 2 mM) to treat C2C12 myotubes for 24 h. Metformin at low concentration (0.5 mM) increased the mRNA expression of myostatin but reduced myostatin expression when higher concentrations were used (Fig. 2A). Consistently, 0.5 mM metformin increased myostatin protein concentration (Fig. 2B) while higher metformin concentrations decreased myotatin protein levels.

3.2. AMPK KO reduced myostatin protein in C2C12 cells

Because chemicals might have nonspecific effects, we further used ectopic expression to test the role of AMPK in myostatin expression in C2C12 cells. We transducted C2C12 cells with lentiviruses carrying a construct expressing AMPK wild-type (AMPK WT) or AMPK mutant (AMPK K45R), respectively. After 36 h following vector exposure, the myostatin protein level in AMPK knockdown cells (AMPK K45R) was much lower compared to control and AMPK WT (Fig. 3A). These data clearly show that AMPK activation increased mRNA and protein levels of myostatin in C2C12 cells (Fig. 3B).

4. Discussion

In skeletal muscle, protein levels are determined by relative rates of protein synthesis and breakdown. The balance between synthesis and degradation of intracellular components determines the overall muscle fiber size. AMPK was recently shown to increase myofibrillar protein degradation through enhancing the expression of MAFbx and MuRF1 [11], but the exact mechanisms linking AMPK to ubiquitin ligase expression and muscle atrophy is unclear. A recent study showed that myostatin is correlated with the expression of MAFbx [24]; because myostatin is a well known inhibitor of muscle growth, we hypothesized that AMPK promotes muscle protein degradation and inhibits muscle growth partially through enhancing myostatin expression. Indeed, we found that AMPK promotes myostatin expression in C2C12 cells.

While AICAR dose dependently increased myostatin expression, the effect of metformin on myostatin expression appears to be more complicated. Low concentration of metformin increased myostatin expression but higher concentration down-regulated myostatin expression and protein level. The possible explanation is that at high levels, metformin over-activates AMPK, which inhibits overall protein synthesis and anabolic metabolism including

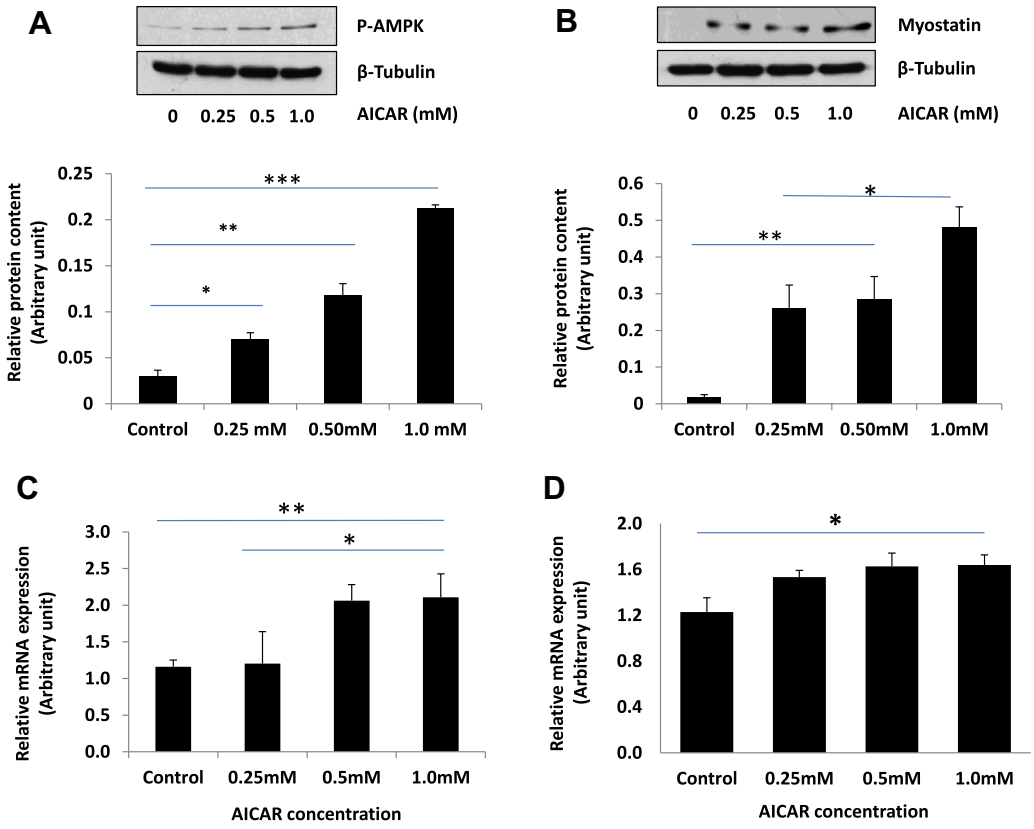

Fig. 1. AMP-activated protein kinase (AMPK) activation stimulated by AICAR induced myostatin mRNA expression in C2C12 myotubes and myoblasts. (A) p-AMPK level in myotubes; (B) myostatin protein concentration inn myotubes; (C) myostatin mRNA expression in myotubes; (D) myostatin mRNA expression in C2C12 myoblasts. C2C12 myoblasts or myotubes were treated with different concentration of AICAR (0, 0.25, 0.5 and 1.0 mM). ($^{**}P < 0.01$; $^{*}P < 0.05$, Mean ± SE; $n = 3$.)

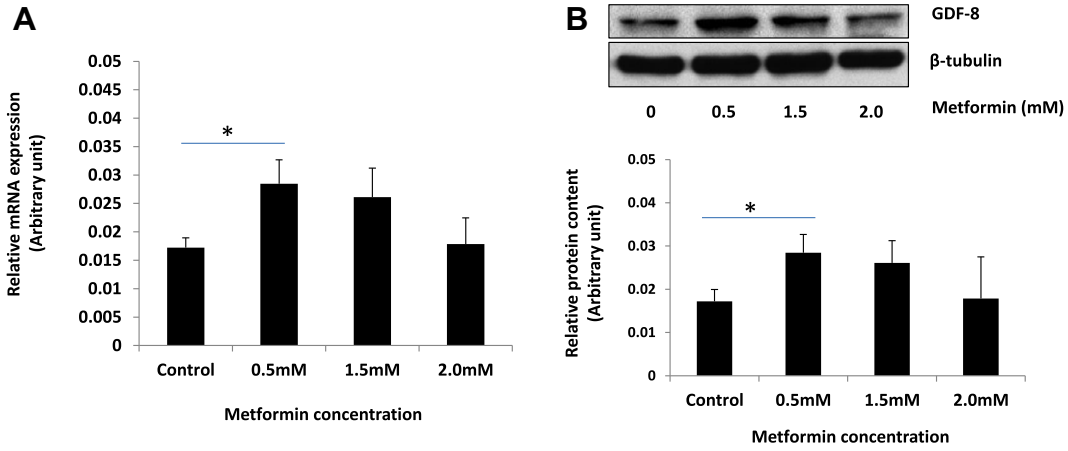

Fig. 2. Metformin enhanced myostatin mRNA expression and protein concentration in C2C12 myotubes. (A) Myostatin mRNA expression; (B) Myostatin protein concentration. C2C12 myoblasts were treated with different concentration of metformin (0, 0.5, 1.5 and 2.0 mM) after inducing myotube formation for 24 h. ($^{*}P < 0.05$, Mean ± SE; $n = 3$.)

myostatin expression. The reduced expression of MSTN may act to protect cells from inhibition by AMPK during myogenesis [25]. To further confirm that AMPK activation plays a key role in myostatin protein level in C2C12 cells, we transducted C2C12 cells with lentiviruses carrying AMPK wild-type to increase or AMPK K45R mutant to knock down AMPK activity. Here, we use a lentiviral vector system due to the high transduction efficiency in C2C12 cells. Lentiviruses efficiently integrate resulting in stable expression, whereas plasmid transfection leads to mostly transient expression with rare cells integrating plasmid DNA leading to stable expression. Consistent with data from chemical activation of AMPK, knocking down AMPK through ectopic expression reduced both myostatin mRNA expression and protein content in myoblasts. In summary, our results show that AMPK activation by AICAR, metformin and ectopic expression induced myostatin mRNA expression and increased its protein level in C2C12 cells.

It is interesting to note, though, the effect of AMPK on myostatin expression appears to be specific to myotubes, not myoblasts. The exact mechanism for such differential effects is unclear, but may be related to the isoform specific roles of AMPK. AMPK α2 subunit is highly expressed in myofibers, and is the major subunit regulating energy metabolism [26,27]. The high abundance of AMPK α2

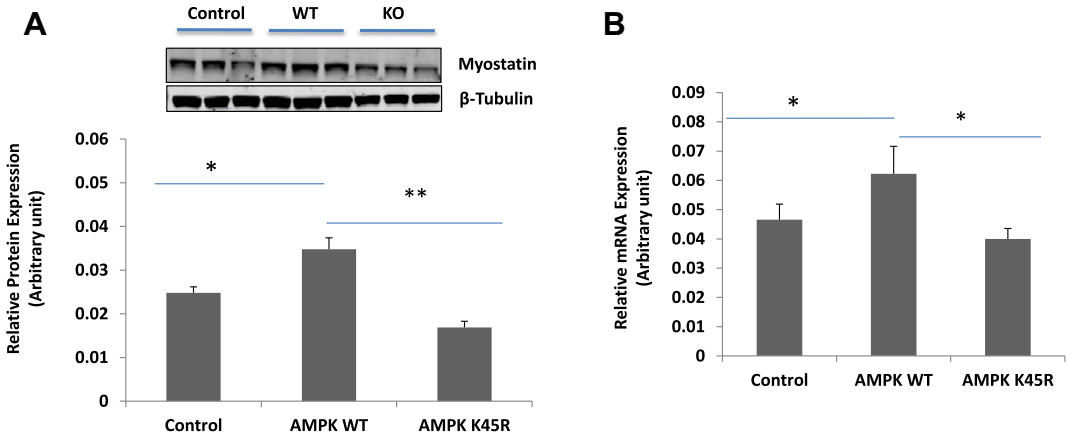

Fig. 3. Myostatin protein concentration and mRNA expression in C2C12 cells ectopically expressing AMPK wild-type (WT) or K45R mutant -subunit. C2C12 cells were transduced with either AMPK-WT or AMPK-K45R lentiviral constructs. (A) Total myostatin protein concentration; (B) Myostatin mRNA expression. (**$P < 0.01$; *$P < 0.05$, Mean ± SE; $n = 3$.)

subunit in myotubes may render it more responsive to environment factors and correlated with the muscle protein homeostasis via myostatin.

To corroborate our finding, recently studies also show that myostatin plays a role in energy metabolism. Myostatin regulates glucose metabolism in C2C12 myotubes *in vitro* and *in vivo*, an effect likely mediated by activating AMPK [28,29]. For the first time, we observed that AMPK induces myostatin expression, establishing the feed-forward loop between AMPK and myostatin to restrict muscle growth. This is an important addition to the current knowledge. Previously, AMPK has been demonstrated to induce cell autophagy [30] and inhibition of mTOR signaling and overall protein synthesis [31]. Our observation hereby provides an additional mechanism for the regulatory role of AMPK in muscle growth. It is novel and important because it establishes the link between AMPK and myostatin, critical pathways regulating energy metabolisms and muscle sizes, respectively.

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