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≪Research Note≫

AMP-activated Protein Kinase Activation Suppresses Protein Synthesis and mTORC1 Signaling in Chick Myotube Cultures

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Protein synthesis in skeletal muscle is considered one of the most energy-consuming cellular processes. AMP-activated protein kinase (AMPK) is a metabolic master switch that regulates glucose and lipid metabolism, and it is implicated in protein synthesis control in skeletal muscles. The mechanistic target of rapamycin complex 1 (mTORC1) is a central regulator of protein metabolism in cells. However, the effect of AMPK activation on protein synthesis and mTORC1 signaling in chicken skeletal muscle remains unclear. Therefore, in this study, we aimed to investigate the effect of 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR), an AMPK activator, on protein synthesis and mTORC1 signaling in chick myotube cultures. The incubation of chick myotubes with AICAR (1 mM) for 3 h led to a significant increase in AMPK (Thr172) phosphorylation. Nonetheless, protein synthesis, measured using the surface sensing of translation method, was significantly decreased by AICAR. In addition, the phosphorylation of p70 ribosomal S6 kinase 1 (S6K1, Thr389), S6 ribosomal protein (Ser240/244), and eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1, Thr37/46) was significantly reduced by AICAR. These results suggest that AMPK activation suppresses protein synthesis and mTORC1 signaling (through the phosphorylation of S6K1, S6 ribosomal protein, and 4E-BP1) in chick myotubes.

Key words: AICAR, AMPK, chick myotubes, mTOR, protein synthesis

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Introduction

AMP-activated protein kinase (AMPK) works as an energy sensor that responds to intracellular AMP:ATP ratio changes (Hardie and Carling, 1997). AMPK activation results in the stimulation of various cellular processes involved in ATP production, such as glucose uptake (Mu *et al*., 2001; Rutter *et al*., 2003) and fatty acid oxidation (Henin *et al*., 1995; Park *et al*., 2002), and in the repression of energy-consuming processes, such as fatty acid (Henin *et al*., 1995) and protein synthesis (Bolster *et al*., 2002; Horman *et al*., 2002; Metayer-Coustard *et al*., 2007). The AMPK-mediated repression of protein synthesis relates to reduced protein kinase signaling, referred to as the mechanistic target of rapamycin (mTOR) (Bolster *et al*., 2002). In pharmacology, 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) is commonly applied to directly activate AMPK without altering the cellular concentrations of ATP, ADP, and AMP (Corton *et al*., 1995). AMPK activation in skeletal muscle, either through endurance exercise or AICAR administration, inhibits protein synthesis and reduces mTOR signaling (Bolster *et al*., 2002; Stephens *et al*., 2002). However, the effect of AMPK activation on protein synthesis and mTOR signaling in chicken skeletal muscle remains to be elucidated.

mTOR, a serine/threonine kinase, regulates essential cellular processes, such as growth, proliferation, differentiation, survival, and metabolism (Laplante and Sabatini, 2012; Saxton and Sabatini, 2017). The mechanistic target of rapamycin complex 1 (mTORC1) is a central regulator of cellular protein metabolism (Ma and Blenis, 2009). mTORC1 stimulates RNA translation by phosphorylating downstream target proteins, such as p70 ribosomal S6 kinase 1 (S6K1) and eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1) (Roux and Topisirovic, 2012). mTORC1-mediated S6K1-activation (phosphorylation of Thr389) leads to the phosphorylation of the S6 ribosomal protein, thereby enhancing the translation of mRNAs containing a 5′-terminal oligopyrimidine tract (Ruvinsky and Meyuhas, 2006). mTORC1-mediated phosphorylation of 4E-BP1 at Thr37/46, Ser65, and Thr70 induces their

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dissociation from eIF4E, thereby allowing their association with eIF4G and the assembly of eIF4F translation initiation complex at the 5′ end of the mRNA (Gingras *et al*., 1999). Furthermore, mTORC1 regulates mRNA translation and induces a potent and rapid increase in the rate of protein synthesis (Wang and Proud, 2006). The skeletal muscle mass is maintained by the balance between the rate of protein synthesis and degradation. Similar to that in other tissues, protein synthesis in skeletal muscle is crucially controlled by the mTOR signaling (Bodine *et al*., 2001). In avian QM7 myoblasts (a quail muscle cell line), AMPK activation also inhibits the S6K1 pathway and protein synthesis (Metayer-Coustard *et al*., 2007). Nevertheless, in chicken skeletal muscles, the mTORC1 signaling-mediated regulation of protein synthesis remains unclear. In addition, mTORC1 signaling, through which AMPK activation prevents the phosphorylation of S6K1, S6 ribosomal protein, and 4E-BP1, is not completely established in chicken skeletal muscles.

The surface sensing of translation (SUnSET) method was developed and validated against radioactive methods for measuring in protein synthesis changes in cultured cells (Schmidt *et al*., 2009; Goodman *et al*., 2011). Therefore, in the current study, we used the SUnSET method to examine how AMPK activation could affect protein synthesis in chick myotubes. We then applied this method to show that AMPK activation could repress protein synthesis and mTORC1 signaling in chick myotube cultures.

In the present study, we demonstrate that AMPK activation suppresses protein synthesis and mTORC1 signaling (through the phosphorylation of S6K1, S6 ribosomal protein, and 4E-BP1) in chick myotube cultures.

Materials and Methods

Antibodies

Antibodies against AMPKα (#2532), phospho-AMPKα (Thr172, #2535), S6K1 (#9202), phospho-S6K1 (Thr389, #9205), S6 ribosomal protein (#2217), phospho-S6 ribosomal protein (Ser240/244, #2215), 4E-BP1 (#9452), and phospho-4E-BP1 (Thr37/46, #9459) were purchased from Cell Signaling Technology (Danvers, MA, USA). The antibody against actin (A2066) was purchased from Sigma Aldrich (St. Louis, MO, USA). The antibody against puromycin (clone 12D10, MABE343) was purchased from Merck Millipore (Burlington, MA, USA).

Cell Culture

Myoblasts were isolated from the thigh muscle of 13-day-old chick embryos as described previously (Nakashima *et al*., 2020b). The embryonic muscle tissue was digested with dispase (Gibco, USA), and the cell suspension was transferred to an uncoated culture dish to allow fibroblast attachment. Cell numbers were counted; then the cells were plated onto gelatin-coated 6-well plates (Iwaki Scitech, Japan) at a density of 2.0×10^5 cells/well. Chick myoblasts were cultured and grown in an M-199 medium containing 15% calf serum and 2.5% chicken embryo extract at 37℃ in a humidified atmosphere of 5% CO_2 in the air during a 7-day incubation period. By day seven, the cells formed myotubes and were incubated for 3 h in serum-free M-199 medium containing AICAR (1 mM, Toronto Research Chemicals, Canada). All experimental procedures were conducted in accordance with the guidelines established by the Animal Care and Use Committee of the National Institute of Livestock and Grassland Science.

Protein Synthesis Measurement

Protein synthesis in chick myotubes was determined as described previously (Nakashima *et al*., 2020) using the SUnSET method, which measures the incorporation of puromycin into nascent peptide chains (Schmidt *et al*., 2009; Goodman *et al*., 2011). Briefly, puromycin was added to the culture treatment media (1 μM final concentration) for 30 min before cell lysis using the RIPA lysis buffer system (Santa Cruz Biotechnology, Dallas, TX, USA). The amount of puromycin incorporated into the newly synthesized protein was determined using western blotting. The membrane was stained with Ponceau S to ensure equal protein loading.

Western Blotting

The cultured cells were washed twice with ice-cold phosphate-buffered saline and lysed using the RIPA lysis buffer system (Santa Cruz Biotechnology). The total protein concentration was estimated using a bicinchoninic acid assay and a commercial kit (Pierce, USA), with bovine serum albumin as a standard.

Western blotting analysis was conducted as described previously (Nakashima and Ishida, 2020a). The lysates with equal amounts of protein were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred onto a nitrocellulose or polyvinylidene difluoride membrane. The membranes were blocked with 5% skim milk in Tris-buffered saline containing 0.1% (v/v) Tween 20 (TBS-T) for 1 h at room temperature. Subsequently, the blocked membranes were incubated with the primary antibody overnight at 4℃. After incubation with the primary antibody, the membrane was incubated with a horseradish peroxidase-conjugated secondary antibody (GE Healthcare, USA) for 1 h at room temperature. To re-probe the western blots, the membranes were stripped with stripping buffer for 30 min at 50℃, washed with TBS-T, and then re-blocked prior to their incubation with the antibody. The bands were visualized using Western Blotting Detection Reagent (GE Healthcare, USA) and a LAS-3000 mini Imaging System (Fujifilm, Japan). The relative band intensity was quantified using ImageJ software (National Institutes of Health, USA).

Statistical Analysis

Data were analyzed using Student's *t*-test. A P-value of <0.05 was considered statistically significant. Data were expressed as the mean±standard error (SE).

Results and Discussion

Figure 1 shows the effects of AICAR on AMPK phosphorylation. AICAR significantly ($P<0.01$) increased the phosphorylation of AMPK (Thr172) in chick myotubes. AMPK is reportedly as a sensor of cellular energy status that

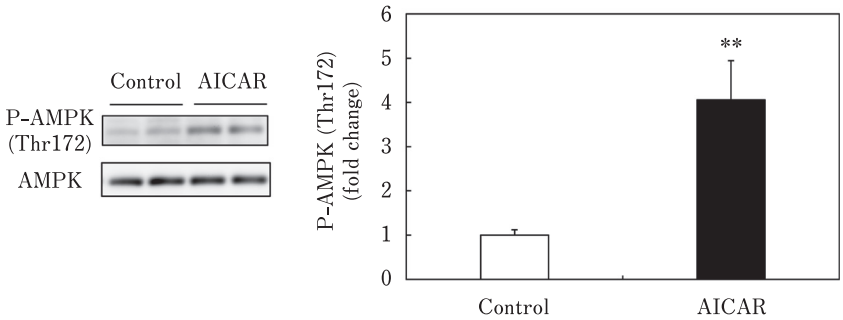

Fig. 1. **Effects of AICAR on AMPK activity [phosphorylation of AMPK (Thr172)] in chick myotube cultures.** The cells were incubated for 3 h in serum-free M-199 medium with AICAR (1 mM). The cell samples were subjected to western blotting analysis using the indicated antibodies. Phosphorylation of proteins, quantitative analysis of western blotting data in cell samples using densitometry scanning. Data are expressed as the mean±SE (n=6). **, $P<0.01$ *versus* the control.

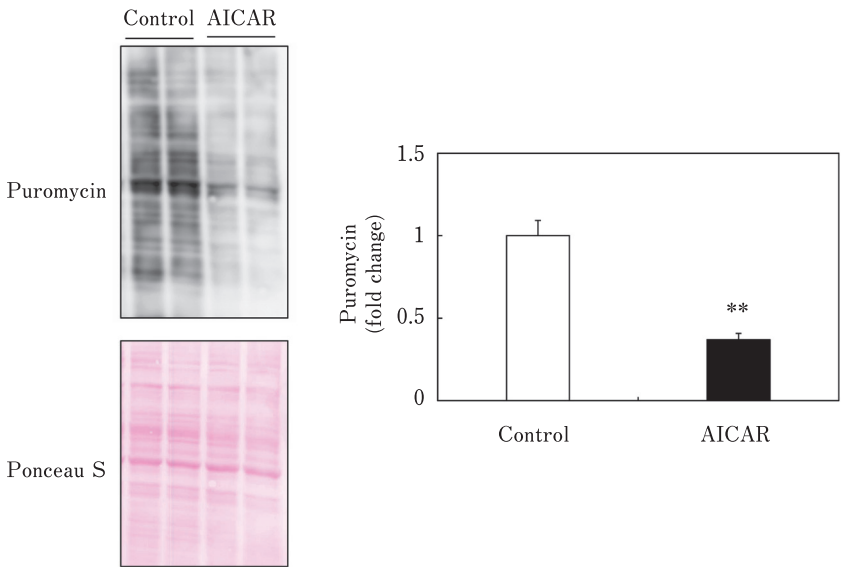

Fig. 2. **Effects of AICAR on protein synthesis in chick myotube cultures.** The cells were incubated for 3 h in serum-free M-199 medium with AICAR (1 mM). The cell samples were subjected to western blotting analysis using an anti-puromycin antibody. A representative image of western blotting for puromycin followed by Ponceau S staining to verify equal loading of protein. Puromycin quantitative analysis of the western blotting data in cell samples using densitometry scanning. Data are expressed as the mean±SE (n=6). **, $P<0.01$ *versus* the control.

regulates reciprocal changes in the levels of AMP and ATP (Hardie and Hawley, 2001). Moreover, AICAR reportedly stimulates AMPK activity through its phosphorylation (Thr172) in mammalian skeletal muscle cell cultures (Williamson *et al*., 2006). AICAR (1-10 mM) stimulates the AMPK activity through its phosphorylation (Thr172) in avian QM7 myoblasts (a quail muscle cell line) (Metayer-Coustard *et al*., 2007). In this study, we revealed that AICAR (1 mM) stimulates AMPK activity through its phosphorylation (Thr172) in primary chick myotube cultures.

Figure 2 shows the effects of AICAR on protein synthesis in chick myotubes, measured using the SUnSET method. AICAR treatment significantly ($P<0.01$) reduced puromycin content, indicating that AICAR inhibits protein synthesis in chick myotube cultures. In the present study, we investigated the direct effects of AICAR, an AMPK activator,

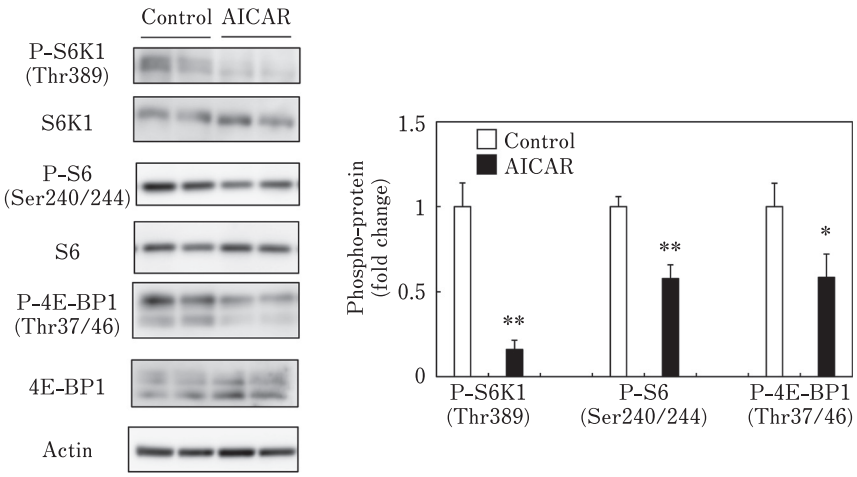

Fig. 3. **Effects of AMPK and mTOR signaling in chick myotube cultures.** The cells were incubated for 3 h in serum-free M-199 medium with AICAR (1 mM). The cell samples were subjected to western blotting analysis using the indicated antibodies. Actin was used as a loading control. Phosphorylation of proteins, quantitative analysis of western blotting data in cell samples using densitometry scanning. Data are expressed as the mean±SE (n=6). **, $P<0.01$, *, $P<0.05$ *versus* the control.

on protein synthesis and aimed to characterize cellular responses involved in this process. The SUnSET method was developed and validated against radioactive methods for measuring protein synthesis changes in cultured cells (Schmidt *et al*., 2009; Goodman *et al*., 2011). We previously reported, using the SUnSET method, that protein synthesis is stimulated by insulin in chick myotubes (Nakashima *et al*., 2020b). The results of the present study also showed that the SUnSET method could be efficiently used to investigate protein synthesis in chick myotubes. Previous studies have found that AMPK activation repressed protein synthesis in skeletal muscle (Bolster *et al*., 2002) and cultured skeletal muscle cells (Bolster *et al*., 2002; Williamson *et al*., 2006). Metayer-Coustard *et al*. (2007) reported that AICAR (1, 2.5, and 5 mM) suppressed protein synthesis in avian QM7 myoblasts (a quail muscle cell line). However, these studies used radioactive methods to measure protein synthesis in skeletal muscle and cultured skeletal muscle cells. In our study, we used the SUnSET method to measure protein synthesis in chick skeletal muscle cells and examined the effect of AICAR (AMPK activation) on protein synthesis. We showed that AICAR inhibits protein synthesis in cultured chicken skeletal muscle cells.

Figure 3 shows the effects of AICAR on mTORC1 signaling in chick myotubes. AICAR significantly decreased the phosphorylation of S6K1 (Thr389) ($P<0.01$) and S6 ribosomal protein (Ser240/244) ($P<0.01$), as well as that of 4E-BP1 (Thr37/46) ($P<0.05$) in primary chick myotube cultures. These results indicate that AMPK activation reduced mTORC1 signaling by affecting the phosphorylation of S6K1 (Thr389), S6 ribosomal protein (Ser240/244), and 4E-BP1 (Thr37/46) in chick myotubes. AICAR stimulates AMPK phosphorylation (i.e., AMPK activity) in both mammalian skeletal muscles (Bolster *et al*., 2002) and cultured skeletal muscle cells (Bolster *et al*., 2002; Williamson *et al*., 2006). Moreover, an association between AICAR-induced reduction in protein synthesis and repression of mTORC1 signaling has been demonstrated (Bolster *et al*., 2002; Reiter *et al*., 2005). AICAR also represses the S6K1 pathway [phosphorylation of S6K1 (Thr389) and S6 ribosomal protein (Ser235/236)] in avian QM7 myoblasts (a quail muscle cell line) (Metayer-Coustard *et al*., 2007). These results are consistent with the results of our study. mTOR stimulates cell growth through the mTORC1 phosphorylation of S6K1 and 4E-BP1 (Roux and Topisirovic, 2012). AMPK activation has also been reported to reduce the phosphorylation of 4E-BP1 in both mammalian skeletal muscles (Bolster *et al*., 2002) and mammalian skeletal muscle cell cultures (Williamson *et al*., 2006). However, the effect of AMPK activation on 4E-BP1 phosphorylation in avian skeletal muscle cell cultures has not yet been reported. In this study, we found that AMPK activation inhibits 4E-BP1 phosphorylation in chick myotubes.

Previously, AMPK activation has been revealed to suppress protein synthesis in both skeletal muscles (Bolster *et al*., 2002) and cultured skeletal muscle cells (Williamson *et al*., 2006; Metayer-Coustard *et al*., 2007). In our study, we demonstrated, using SUnSET methods, that AMPK activation suppressed protein synthesis in chick skeletal muscle cells. Furthermore, AMPK activation has previously been shown to control mTORC1 signaling in skeletal muscles (Deshmukh *et al*., 2008). In our study, we demonstrated that AMPK activation inhibited mTORC1 signaling by affecting the phosphorylation of S6K1, S6 ribosomal protein,

and 4E-BP1 in chick skeletal muscle cells. These findings indicate that AMPK activation suppressed protein synthesis through mTORC1 signaling in chicken skeletal muscle. Nevertheless, as our study was performed in cultured chicken cells, it does not supply direct data on the *in vivo* conditions in chicken skeletal muscles. Therefore, future investigations should focus on the effect of AICAR using *in vivo* model systems.

In summary, this study demonstrates that AMPK activation suppresses protein synthesis and mTORC1 signaling by modulating the phosphorylation of S6K1, S6 ribosomal protein, and 4E-BP1 in chick myotubes.

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Conflicts of Interest

The authors declare no conflict of interest.

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