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

Kazuki Nakashima and Aiko Ishida
Division of Animal Metabolism and Nutrition, Institute of Livestock and Grassland Science, NARO, Tsukuba 305-0901, Japan

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
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°C in a humified atmosphere of 5% CO₂ 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°C. 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°C, 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
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 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.
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.

References

Bodine SC, Stitt TN, Gonzalez M, Kline WO, Stover GL, Bauerlein R, Zlotchenko E, Scrimgeour A, Lawrence JC, Glass DJ and Yanopoulos GD. Akt/mTOR pathway is a crucial regulator of skeletal muscle hypertrophy and can prevent muscle atrophy in vivo. Nature Cell Biology, 3: 1014–1019. 2001.

Bolster DR, Crozier SJ, Kimball SR and Jefferson LS. AMP-activated protein kinase suppresses protein synthesis in rat skeletal muscle through down-regulated mammalian target of rapamycin (mTOR) signaling. Journal of Biological Chemistry, 277: 23977–23980. 2002.

Corton JM, Gillespie JG, Hawley SA and Hardie DG. 5-aminoimidazole-4-carboxamide ribonucleoside. A specific method for activating AMP-activated protein kinase in intact cells? European Journal of Biochemistry, 229: 558–565. 1995.

Deshmukh AS, Treebak JT, Long VC, Viollet B, Wojtaszewski JF and Zierath JR. Role of adenosine 5′-monophosphate-activated protein kinase subunits in skeletal muscle mammalian target of rapamycin signaling. Molecular Endocrinology, 22: 1105–1112. 2008.

Gingras AC, Gygi SP, Raught B, Polakiewicz RD, Abraham RT, Rabinowitz JD, Roux PP and Topisirovic I. Regulation of mRNA translation by AMPK. European Journal of Biochemistry, 253: 431–438. 1997.

Goodman CA, Mabrey DM, Frey JW, Miu MH, Schmidt EK, Pierre P and Hornberger TA. Novel insights into the regulation of skeletal muscle protein synthesis as revealed by a new nonradioactive in vivo technique. FASEB Journal, 25: 1028–1039. 2011.

Hardie DG and Carling D. The AMP-activated protein kinase - fuel gauge of the mammalian cell? European Journal of Biochemistry, 246: 259–273. 1997.

Hardie DG and Hawley SA. AMP-activated protein kinase: the energy charge hypothesis revisited. Bioessays, 23: 1112–1119. 2001.

Henin N, Vincent MF, Gruber HE and Van den Berghhe G. Inhibition of fatty acid and cholesterol synthesis by stimulation of AMP-activated protein kinase. FASEB Journal, 9: 541–546. 1995.

Horman S, Browne G, Krause U, Patel J, Vertommen D, Bertrand L, Lavoinne A, Hue L, Proud C and Rider M. Activation of AMP-activated protein kinase leads to the phosphorylation of elongation factor 2 and an inhibition of protein synthesis. Current Biology, 12: 1419–1423. 2002.

Laplante M and Sabatini DM. mTOR signaling in growth control and disease. Cell, 149: 274–293. 2012.

Ma XM and Blenis J. Molecular mechanisms of mTOR-mediated translational control. Nature Review Molecular Cell Biology, 10: 307–318. 2009.

Metayer-Coustard S, Collin A, Audouin E, Crochet S, Dupont J and Tesserault S. AMPK regulates the S6K1 pathway and protein synthesis in avian QM7 myoblasts. In: Energy and Protein Metabolism and Nutrition (EAAP Publication ed.). No. 124. pp. 181–182. Wageningen Academic Publisher. 2007.

Mu J, Brozinick JT Jr., Valladares O, Bucan M and Birnbaum MJ. A role for AMP-activated protein kinase in contraction- and hypoxia-regulated glucose transport in skeletal muscle. Molecular Cell, 7: 1085–1094. 2001.

Nakashima K and Ishida A. Regulation of autophagy in chick skeletal muscle: effect of mTOR inhibition. Journal of Poultry Science, 57: 77–83. 2020a.

Nakashima K, Ishida A, Shimamoto S, Ijiri D and Ohutsuka A. Insulin stimulation of protein synthesis and mTOR signaling in chick myotube cultures. Journal of Poultry Science, 57: 205–209. 2020b.

Park H, Kaushik VK, Constant S, Pretounti M, Przybytkowski E, Ruderman NB and Saha AK. Coordinate regulation of malonyl-CoA decarboxylase, sn-glycerol-3-phosphate acyltransferase, and acetyl-CoA carboxylase by AMP-activated protein kinase in rat tissues in response to exercise. Journal of Biological Chemistry, 277: 32571–32577. 2002.

Reiter AK, Bolster DR, Crozier SJ, Kimball SR and Jefferson LS. Repression of protein synthesis and mTOR signaling in rat liver mediated by the AMPK activator aminomidazole ribonucleoside. American Journal of Physiology Endocrinology and Metabolism, 288: E980–E988. 2005.

Roux PP and Topisirovic I. Regulation of mRNA translation by signaling pathways. Cold Spring Harbor Perspectives Biology, 4: a012252. 2012.

Rutter GA, Da Silva Xavier G and Leclerc I. Roles of 5′-AMP in mTOR signalling. Nature, 499: 217–221. 2013.

Schmidt EK, Clavarino G, Ceppi M and Pierre P. SUnSET, a nonradioactive method to monitor protein synthesis. Nature, 405: 1014–1019. 2000.

Ruderman NB and Saha AK. AMP-activated protein kinase (AMPK) in mammalian glucose homoeostasis. Biochemical Journal, 375: 1–16. 2003.

Ruvinsky I and Meyuhas O. Ribosomal protein S6 phosphorylation: from protein synthesis to cell size. Trends in Biochemical Sciences, 31: 342–348. 2006.

Saxton RA and Sabatini DM. mTOR Signaling in Growth, Metabolism, and Disease. Cell, 168: 960–976. 2017.

Schmidt EK, Clavarino G, Ceppi M and Pierre P. SUnSET, a nonradioactive method to monitor protein synthesis. Nature Methods, 6: 275–277. 2009.

Stephens TJ, Chen ZP, Canny BJ, Mitchel BJ, Kemp BE and McConell GK. Progressive increase in human skeletal muscle AMPKα2 activity and ACC phosphorylation during exercise. American Journal of Physiology Endocrinology and Metabolism, 282: E688–E694. 2002.

Wang X and Proud CG. The mTOR pathway in the control of protein synthesis. Physiology (Bethesda), 21: 362–369. 2006.

Williamson DL, Bolster DR, Kimball SR and Jefferson LS. Time course changes in signaling pathways and protein synthesis in C2C12 myotubes following AMPK activation by AICAR. American Journal of Physiology Endocrinology and Metabolism, 291: E80–E89. 2006.