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≪Research Note≫

Insulin Stimulation of Protein Synthesis and mTOR Signaling in Chick Myotube Cultures

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Insulin stimulates protein synthesis in skeletal muscles. Protein synthesis is controlled by the mechanistic target of rapamycin (mTOR) signaling in skeletal muscles. This study was conducted to investigate the effect of insulin on protein synthesis and mTOR signaling in chick myotube cultures. Chick myotubes were incubated with insulin (1 μg/m*l*) for 1 h. Protein synthesis, measured using the surface sensing of translation method, was significantly increased by insulin. The phosphorylation of AKT (Thr308 and Ser473), p70 ribosomal S6 kinase 1 (S6K1, Thr389), S6 ribosomal protein (Ser235/236), and eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1, Thr37/46) was also significantly increased by insulin. These results suggest that insulin stimulates protein synthesis via mTOR signaling (phosphorylation of AKT, S6K1, S6 ribosomal protein, and 4E-BP1) in chick myotube cultures.

Key words: AKT, chick myotubes, insulin, mechanistic target of rapamycin, protein synthesis

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Introduction

The mechanistic target of rapamycin (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). mTOR is a central regulator of protein metabolism in cells (Ma and Blenis, 2009). mTOR kinase assembles into two distinct complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2), which differ in their composition, substrates, functions, and sensitivity to the inhibitor rapamycin (Laplante and Sabatini, 2012; Saxton and Sabatini, 2017). 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). Activation of S6K1 (phosphorylation of Thr389) by mTORC1 phosphorylates the S6 ribosomal protein, and this enhances the translation of mRNAs containing a 5′-terminal oligopyrimidine tract (Ruvinsky and Meyuhas, 2006). Phosphorylation of 4E-BP1 at Thr37/46, Ser65, and Thr70 by mTORC1 induces their dissociation from eIF4E, thereby allowing their association with eIF4G and the assembly of the eIF4F translation initiation complex at the 5' end of mRNA (Gingras *et al*., 1999). mTORC2 controls cell migration, cytoskeleton rearrangement, and gluconeogenesis by phosphorylating several substrates, including AKT at Ser473 (Sarbassov *et al*., 2005). Furthermore, mTOR regulates the translation of mRNA, and induces a potent and rapid increase in the rate of protein synthesis (Wang and Proud, 2006).

Skeletal muscle mass is maintained by the balance between the rate of protein synthesis and protein degradation. Similar to other tissues, protein synthesis in skeletal muscle is crucially controlled by mTOR signaling (Bodine *et al*., 2001). However, in chicken skeletal muscles, the regulation of protein synthesis by mTOR signaling remains unclear.

Insulin has an important role in growth in animals and regulates protein synthesis and degradation in skeletal muscles, with the latter causing muscle hypertrophy (Prod'homme *et al*., 2004; Liu *et al*., 2006). Insulin also stimulates mTOR signaling in mammalian skeletal muscles (Shah *et al*., 2000) and stimulates protein synthesis via this same signaling in skeletal muscles (Davis *et al*., 2002; Timmerman *et al*., 2010) and cultured skeletal muscle cells (Kimball *et al*., 1998; Shen *et al*., 2005). Although the regulatory effects of insulin on protein synthesis and mTOR signaling in mammalian skeletal muscles are well known, these effects in chicken skeletal muscles are largely unknown. In addition,

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the mTOR signaling through which insulin stimulates the phosphorylation of AKT, S6K1, S6 ribosomal protein, and 4E-BP1 in chicken skeletal muscles is not fully understood.

In the present study, we demonstrate that insulin stimulates protein synthesis and mTOR signaling (phosphorylation of AKT, S6K1, S6 ribosomal protein, and 4E-BP1) in chick myotube cultures.

Materials and Methods

Antibodies

Antibodies against AKT (#9272), phospho-AKT (Ser473, #9271), phospho-AKT (Thr308, #2965), S6K1 (#9202), phospho-S6K1 (Thr389, #9205), S6 ribosomal protein (#2217), phospho-S6 ribosomal protein (Ser235/236, #2211), 4E-BP1 (#9452), and phospho-4E-BP1 (Thr37/46, #9459) were purchased from Cell Signaling Technology (Danvers, MA, USA). The antibody against α-tubulin (T9026) 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. The muscle tissue obtained from the embryos were digested with dispase (Life Technologies, Carlsbad, CA, USA), and the cell suspension was transferred to an uncoated culture dish to allow fibroblast attachment. Cell numbers were counted, and the cells were then plated onto gelatin-coated 6-well plates (AGC Techno Glass Co., Yoshida, Shizuoka, Japan) at a density of 2.0×10^5 cells/well. Chick myoblasts were cultured in M-199 medium containing 15% calf serum and 2.5% chicken embryo extract and were grown at 37℃ in a humidified atmosphere of 5% CO_2 in air during a 7-day incubation period. On day seven, the cells had formed myotubes and were incubated for 1 h in serum-free M-199 medium containing insulin (1 μg/m*l*, from porcine pancreas; Sigma Aldrich). 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 previously described, using the surface sensing of translation (SUnSET) method, which measures the incorporation of puromycin into nascent peptide chains (Schmidt *et al*., 2009; Goodman *et al*., 2011). In brief, puromycin was added to the culture treatment media (1 μM final concentration) for 30 min before cell lysis in 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 loading of the protein.

Western Blotting

Cultured cells were washed twice with ice-cold phosphate-buffered saline (PBS) and lysed in RIPA lysis buffer system (Santa Cruz Biotechnology). The total protein concentration was estimated using a bicinchoninic acid assay and a commercial kit (Life Technologies), with bovine serum albumin as a standard. The lysates with equal amounts of protein were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to 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, Chicago, IL, 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 incubation with the antibody. The bands were visualized using Western Blotting Detection Reagent (GE Healthcare) and a LAS-3000 mini imaging system (Fujifilm, Tokyo, 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 indicative of statistical significance. Data were expressed as the mean±standard deviation (SD).

Results and Discussion

The effect of insulin on protein synthesis in chick myotubes is shown in Fig. 1. Puromycin content was significantly ($P<0.01$) increased, which indicated that insulin stimulated protein synthesis in chick myotube cultures. Previous studies have found that insulin stimulates protein synthesis in cultured mammalian skeletal muscle cells (Kimball *et al*., 1998; Shen *et al*., 2005), and cultured chicken skeletal muscle cells (Airhart *et al*., 1982). However, these studies used radioactive-based methods to measure protein synthesis in cultured skeletal muscle cells. In addition, a previous study used the SUnSET method to demonstrate that insulin stimulates protein synthesis in C2C12 myotubes (Goodman *et al*., 2011). Recently, in chicken myoblast cultures, insulin stimulated protein synthesis for 6 h using the SUnSET method (Wang *et al*., 2019). In the present study, the SUnSET method was used to measure the increment of protein synthesis by insulin for 1 h in chick myotube cultures.

The effect of insulin on mTOR signaling is shown in Fig. 2. Insulin significantly increased the phosphorylation of AKT at Thr308 and Ser473 ($P<0.01$), of S6K1 (Thr389) and S6 ribosomal protein (Ser235/236) ($P<0.01$), and that of 4E-BP1 (Thr37/46) ($P<0.05$). These results indicate that insulin stimulated AKT (Thr308 and Ser473) and mTOR signaling (phosphorylation of AKT (Ser473), S6K1 (Thr 389), ribosomal protein S6 (Ser235/236), and 4E-BP1 (Thr 37/46)) in chick myotubes. Insulin stimulates the phosphorylation of AKT in mammalian skeletal muscles (Suryawan *et al*., 2007; Timmerman *et al*., 2010) and cultured skeletal muscle cells (Krützfeldt *et al*., 2000). Duchene *et al*. (2008) has reported that insulin also stimulates AKT phosphorylation (Ser473) in chicken skeletal muscles and chicken myoblast cultures. This result is consistent with the results

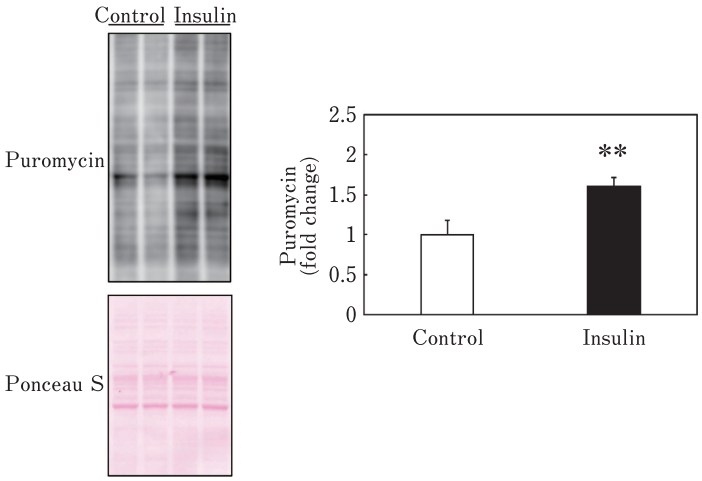

Fig. 1. **The effects of insulin on protein synthesis in chick myotube cultures.** Cells were incubated for 1 h in serum-free M-199 medium with insulin (1 μg/m*l*). Cell samples were subjected to western blot analysis using an anti-puromycin antibody. A representative image of western blotting for puromycin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

of this study. Because AKT at Ser473 is phosphorylated by mTORC2 (Sarbassov *et al*., 2005), this finding indicates that insulin stimulates mTORC2 activity in chicken skeletal muscles. The effect of insulin on the phosphorylation of AKT (Thr308) in chicken skeletal muscle cell cultures was also examined, and it was found that insulin also stimulated the phosphorylation of AKT (Thr308) in chick myotubes. Duchene *et al*. (2008) reported that insulin stimulates AKT phosphorylation (Thr308) in chicken skeletal muscles. However, the effect of insulin on the phosphorylation of AKT (Thr308) in chicken skeletal muscle cell cultures has not been reported. In this study, it was found that insulin stimulates the phosphorylation of AKT (Thr308) in chicken skeletal muscle cell cultures. The full activation of AKT requires phosphorylation on two sites (Thr308 and Ser473), Thr308 by 3-phosphainositide-dependent protein kinase 1 (PDK1) and Ser473 by mTORC2 (Guertin and Sabatini, 2007). In the present study, insulin phosphorylated AKT at both these sites (Thr308 and Ser473) in the chick myotubes, indicating that insulin stimulates the full activation of AKT in chicken skeletal muscles.

mTOR stimulates cell growth through the mTORC1-mediated phosphorylation of S6K1 and 4E-BP1 (Roux and Topisirovic, 2012) and the mTORC2-mediated phosphorylation of AKT (Ser473) (Sarbassov *et al*., 2005). Upon activation by mTORC1, S6K1 phosphorylates S6 ribosomal protein (Ruvinsky and Meyuhas, 2006) to stimulate mRNA translation (Thoreen *et al*., 2012). S6K1 (Thr389) and 4E-BP1 (Thr37/46) are the target proteins of mTORC1 kinase in cells. In the present study, insulin stimulated the phosphorylation of S6K1 and 4E-BP1 in chick myotubes. It has been reported that insulin stimulates the phosphorylation of S6K1 (Duchene *et al*., 2008; Wang *et al*., 2019) and S6 ribosomal protein (Duchene *et al*., 2008) in chicken myoblast cultures. These results are consistent with the results of the present study. Duchene *et al*. (2008) reported that insulin stimulates the phosphorylation of 4E-BP1 in chicken skeletal muscles. However, the effect of insulin on the phosphorylation of 4E-BP1 in chicken skeletal muscle cell cultures has not been reported. In this study, it was found that insulin stimulates the phosphorylation of 4E-BP1 in chick myotubes. Protein synthesis is associated with an increase in the phosphorylation of S6K1 and 4E-BP1, two key regulatory proteins involved in the initiation of mRNA translation (Wang and Proud, 2006). Numerous studies have reported that S6K1 and 4E-BP1 play key roles in the control of insulin-dependent protein synthesis in skeletal muscles (Shah *et al*., 2000; Bolster *et al*., 2004). In the present study, insulin stimulated the phosphorylation of S6K1 and 4E-BP1 in chick myotubes. Both S6K1 and 4E-BP1 are downstream target proteins (substrates) in signaling involving mTOR kinase. The regulation of mTOR signaling (phosphorylation of AKT, S6K1, S6 ribosomal protein, and 4E-BP1) is poorly documented in non-mammalian species. In this study, it was found that insulin stimulates AKT (Thr308) (upstream of mTORC1), AKT (Ser473) (upstream of mTORC1 and substrate of mTORC2), S6K1 (substrate of mTORC1), S6 ribosomal protein (downstream of S6K1) and 4E-BP1 (substrate of mTORC1) in chick myotubes.

In conclusion, this study shows that insulin stimulates protein synthesis via mTOR signaling (phosphorylation of AKT, S6K1, S6 ribosomal protein, and 4E-BP1) in chick

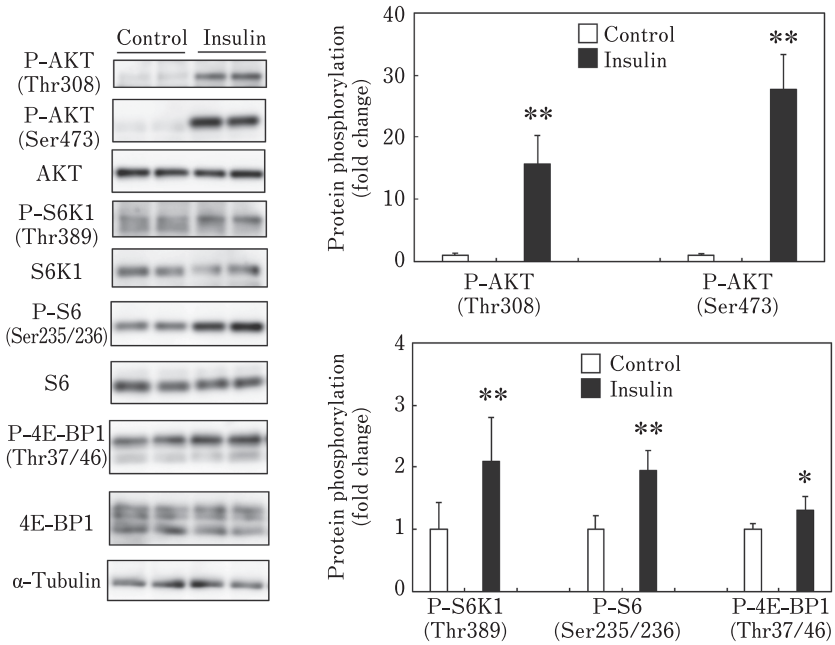

Fig. 2. **The effects of insulin on AKT and mTOR signaling in chick myotube cultures.** Cells were incubated for 1 h in serum-free M-199 medium with insulin (1 μg/m*l*). Cell samples were subjected to western blot analysis using the indicated antibodies. α-Tubulin was used as a loading control. Phosphorylation of proteins, quantitative analysis of the western blot data in cell samples using densitometry scanning. Data are expressed as the mean±SD (n=6). **, $P<0.01$, *, $P<0.05$ *versus* the control.

myotube cultures.

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Conflicts of Interest

The authors declare no conflict of interest.

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