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Regulation of Autophagy in Chick Skeletal Muscle: Effect of mTOR Inhibition

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Autophagy in the skeletal muscle increases under catabolic conditions resulting in muscle atrophy. This study investigated the effect of inhibition of mechanistic target of rapamycin (mTOR) on autophagy in chick skeletal muscle. We examined the effects of Torin1, an mTOR inhibitor, on autophagy. Chick myotubes were incubated with Torin1 (100 nM) for 3 h. It was observed that Torin1 inhibited the phosphorylation of AKT (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), which are used for measurement of mTOR activity. Torin1 significantly ($P<0.01$) increased the LC3-II/LC3-I ratio, an index for autophagosome formation, while it did not influence the expression of autophagy-related genes (LC3B, GABARAPL1, and ATG12). In addition, Torin1 increased atrogin-1/MAFbx (a muscle-specific ubiquitin ligase) mRNA expression. Fasting for 24 h inhibited the phosphorylation of AKT (Ser473), S6K1 (Ther389), S6 ribosomal protein (Ser235/236), and 4E-BP1 (Thr37/46) in chick skeletal muscle and significantly ($P<0.01$) increased the LC3-II/LC3-I ratio. Fasting also increased GABARAPL1 and atrogin-1/MAFbx mRNA expression but not LC3B or ATG12 mRNA expression. These results indicate that mTOR signaling regulates autophagy and the ubiquitin-proteasome proteolytic pathway in chick skeletal muscle.

Key words: atrogin-1/MAFbx, autophagy, chick myotubes, chick skeletal muscle, mechanistic target of rapamycin, Torin1

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Introduction

The mechanistic target of rapamycin (mTOR) signaling pathway is a major regulator of cell growth and protein metabolism. mTOR protein kinase controls cell growth by regulating protein synthesis machinery, autophagy, and the ubiquitin-proteasome proteolytic pathway (Zhao *et al*., 2007; Zhao *et al*., 2015). The mTOR signaling pathway plays a crucial role in regulating autophagy in mammalian cells (Jung *et al*., 2010). However, in chickens, the relationship between the mTOR signaling pathway and autophagy remains unclear.

In skeletal muscles, cell mass is determined by calculating between the rate of protein synthesis and degradation. Animal experiments have consistently demonstrated that protein degradation, via the ubiquitin-proteasome proteolytic pathway (Lecker *et al*., 2004) and autophagy (Sandri, 2010; Sandri, 2013) increase in skeletal muscles undergoing atrophy.

Macroautophagy, hereafter referred to as autophagy, is a cellular lysosomal protein degradation pathway and a bulk degradative process involving protein aggregates and organelles. During autophagy, cytosolic materials, such as proteins and organelles are enveloped by autophagosomes. The autophagosomes fuse with lysosomes, and through the action of lysosomal hydrolases, they are converted into autolysosomes (Mizushima, 2007; Tooze and Yoshimori, 2010). Autophagy provides amino acids, which can be used in protein synthesis, energy production, and gluconeogenesis, in both physiological and nutritional conditions (Kuma *et al*., 2004; Kuma and Mizushima, 2010). In addition, autophagy is essential for the maintenance of skeletal muscle mass under catabolic conditions (Masiero *et al*., 2009; Seiliez *et al*., 2010). Recent studies have shown that autophagy is regulated by the mTOR signaling pathway in mammalian skeletal muscle cells (Zhao *et al*., 2007; Zhao *et al*., 2015). However, the molecular regulation of autophagy by mTOR

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in chicken skeletal muscles has not been reported.

Muscle proteolysis in catabolic conditions is primarily due to activation of the ubiquitin-proteasome proteolytic pathway (Lecker *et al*., 2004), whereby proteins destined for degradation are bound to a chain of ubiquitin molecules, which marks the proteins for rapid breakdown by the proteasomes (Glickman and Ciechanover, 2002). Several studies have suggested that atrogin-1, an E3 ubiquitin ligase referred to as MAFbx (muscle atrophy F-box), plays a pivotal role in muscle atrophy (Bodine *et al*., 2001; Gomes *et al*., 2001). Its expression increases in catabolic conditions, which results in muscle atrophy (Bodine *et al*., 2001; Gomes *et al*., 2001). However, the factors and mechanisms regulating the ubiquitin-proteasome proteolytic pathway (atrogin-1/MAFbx expression) in skeletal muscle are poorly understood. Zhao *et al*. (2015) recently reported that mTOR inhibition promotes the ubiquitin-proteasome proteolytic pathway and autophagy in mammalian myotubes. However, the effect of mTOR inhibition on the ubiquitin-proteasome proteolytic pathway and autophagy in chicken skeletal muscle cells has not yet been examined.

Therefore, this study investigated the effect of mTOR inhibition on autophagy in chick myotubes and chicken skeletal muscle. mTOR inhibition can be archieved in various ways; for example, treatment with inhibitor (Torin1) *in vitro* (Thoreen *et al*., 2009) and fasting *in vivo* (Naito *et al*., 2013). The present study demonstrated that mTOR inhibition promotes autophagy and the ubiquitin-proteasome pathway in chicken skeletal muscle.

Materials and Methods

Cell Culture

Myoblasts were isolated from the thigh muscles of 13-day-old chick embryos (Nakashima *et al*., 1998). Briefly, the muscle tissue obtained from the embryo was digested with dispase (Gibco, USA), and the cell suspension was transferred to an uncoated culture dish to allow for fibroblast attachment. Cell numbers were counted, and the cells were then plated onto gelatin-coated 6-well plates (Iwaki SciTech, Japan) at a density of 2.0×10^5 cells/well. The chicken embryo extract was prepared from 10-day chick embryos. The embryos were rinsed with phosphate-buffered saline (PBS), passed through a 50 mL syringe, and an equal volume of PBS was added. The supernatant collected by centrifugation (10,000 rpm for 1 h) at 4℃ was used as the chicken embryo extract. 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 for 7 days. On day 7, the cells had formed myotubes and were incubated for 3 h in serum-free M-199 medium containing Torin1 (100 nM, Cell Signaling Technology, USA). Torin1 is an ATP-competitive inhibitor that targets the kinase domain of mTOR (Thoreen *et al*., 2009).

Animal Preparation and Experimental Protocol

One-day-old male broiler chicks (chunky) were supplied by a local commercial hatchery (Komatsu Hatchery, Japan) and were housed in an electrically heated battery brooder where they were provided with water and a commercial starter diet (Toyohashi Co. Ltd., Japan) *ad libitum* for 11 days. On day 11, birds with similar body weights were selected and housed in wire-bottomed aluminum cages where they were provided free access to a commercial starter diet and water for 3 days. On day 14, the chicks were divided into two groups: the fed group and the food-deprived group. Fed chickens were maintained as described above. Food-deprived chicks had food removed from their cages 24 h before they were killed. The pectoralis muscle was rapidly excised, frozen in liquid nitrogen, and stored at −80℃ until further use. 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.

Real-time Polymerase Chain Reaction (PCR)

Total RNA was extracted from the pectoralis muscle and chick myotubes using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. Complementary DNA was synthesized from total RNA using a random primer (TaKaRa, Japan) and ReverTra Ace (Toyobo, Japan). The sequences of the primers were as follows: atrogin-1/MAFbx, forward: 5′-CCAACAACCCAGAGACCTGT-3′ and reverse: 5′-GGAGCTTCACACGAACATGA-3′; microtubule-associated protein 1 light chain 3B (LC3B), forward: 5′-TCCGAGATCAGCATCCAACT-3′ and reverse: 5′-CACCATGCTGTGTCCGTTC-3′; GABA (A) receptor-associated protein like 1 (GABARAPL1), forward: 5′-CCGACAGAGTCCCCGTAATT-3′ and reverse: 5′-ATGGTAGCACTTGTGGGAGG-3′; autophagy-related 12 (ATG12), forward: 5′-CGGAAAGGACCCCAGAGAG-3′ and reverse: 5′-CTTGATGAAGTCGCACAGGC-3′; 18S rRNA, forward: 5′-AAACGGCTACCACATCCAAG-3′ and reverse: 5′-CCTCCAATGGATCCTCGTTA-3′. The mRNA levels were measured by real-time PCR using a LightCycler® instrument (Roche Diagnostics, Germany) and the QuantiTect SYBR Green PCR system (Qiagen, Japan). 18S rRNA was used as an internal control.

Immunoblotting

The cells were washed twice with ice-cold PBS and lysed in the RIPA lysis buffer system (Santa Cruz Biotechnology, USA). The lysate was centrifuged at 14,000 rpm for 5 min at 4℃, and the supernatant was collected. The pectoralis muscle of chicks was also homogenized in the RIPA lysis buffer system (Santa Cruz Biotechnology, USA). The homogenate was centrifuged at 10,500 rpm for 10 min at 4℃, and the supernatant was collected. Total protein concentration was estimated by the bicinchoninic acid (BCA) assay using a commercial kit (Pierce, USA) with bovine serum albumin as the standard.

Equal amounts of the protein lysates were separated by 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. The blocked membranes were then incubated with primary antibody

overnight at 4℃, followed by incubation with a horseradish peroxidase-conjugated secondary antibody (GE Healthcare, USA) for 1 h at room temperature. The bands were visualized using Western Blotting Detection Reagent (GE Healthcare, USA) and a LAS-3000 mini image analyzer (Fujifilm, Japan). The relative band intensity was quantified using ImageJ software (National Institutes of Health, USA). To reprobe immunoblots, membranes were stripped with stripping buffer for 30 min at 50℃, washed with TBS-T, and then re-blocked prior to incubation with antibody. Antibodies against LC3B (#2775; 1:2,000 dilution), AKT (#9272; 1:2,000 dilution), phospho-AKT (Ser473; #9271; 1:2,000 dilution), S6 ribosomal protein (#2217; 1:2,000 dilution), phospho-S6 ribosomal protein (Ser235/236; #2211; 1:2,000 dilution), eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1; #9452; 1:2,000 dilution), and phospho-4E-BP1 (Thr37/46; #9459; 1:2,000 dilution) were purchased from Cell Signaling Technology (USA). Antibodies against p70 ribosomal S6 kinase 1 (S6K1; sc-230; 1:2,000 dilution) and phospho-S6K1 (Thr389; sc-11759-R; 1:2,000 dilution) were purchased from Santa Cruz Biotechnology (USA), while the antibody against actin (A2066; 1:5,000 dilution) was purchased from Sigma-Aldrich (USA).

Statistical Analysis

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

Results and Discussion

The effects of Torin1 on mTOR signaling pathway are illustrated in Fig. 1A. The phosphorylation of AKT (Ser 473), S6K1 (Thr389), S6 ribosomal protein (Ser235/236), and 4E-BP1(Thr37/46) was inhibited by Torin1, indicating that Torin1 inhibited AKT and mTOR signaling (phosphorylation of S6K1, S6 ribosomal protein, and 4E-BP1). Immunoblot analysis confirmed the inhibition of the mTOR signaling pathway in chick myotubes. The function of mTOR is to stimulate cell growth through mTOR complex 1 (mTORC1)-mediated phosphorylation of S6K1 and 4E-BP1 (Burnett *et al*., 1998) and mTOR complex 2 (mTORC2)-mediated phosphorylation of AKT (Ser473) (Sarbassov *et al*., 2005). Upon activation by mTORC1, S6K1 phosphorylates S6

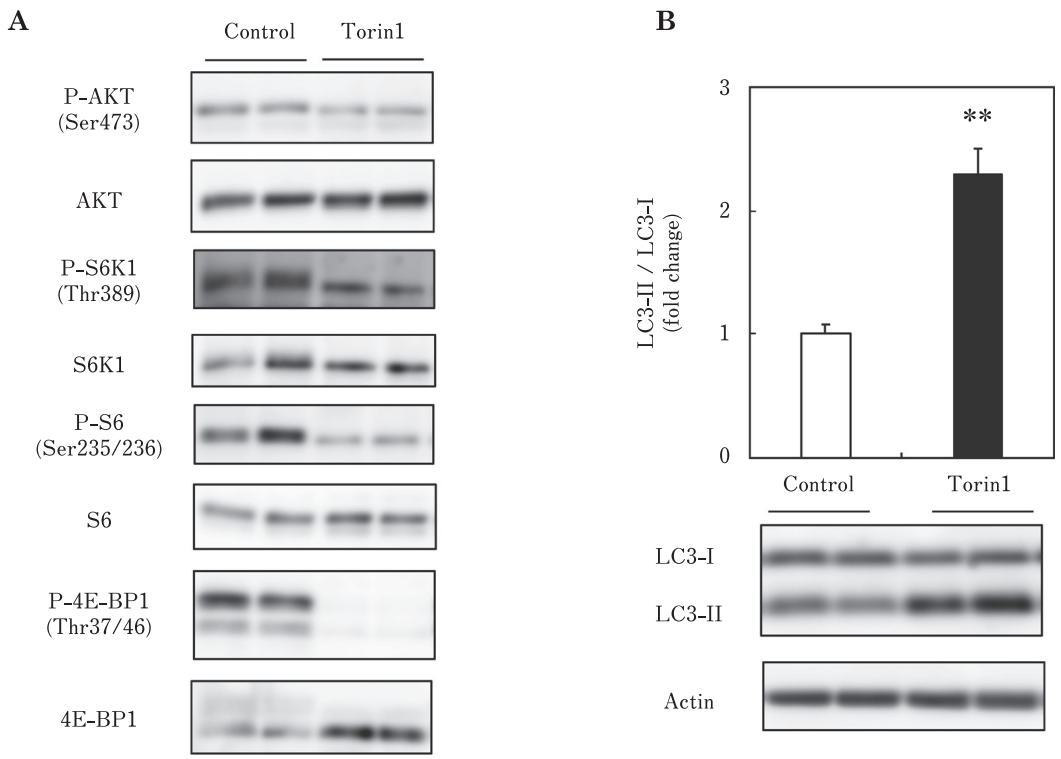

Fig. 1. **Effects of Torin1 on AKT and mTOR signaling and autophagy in chick myotubes.** A, cells were incubated for 3 h in serum-free M-199 medium with Torin1 (100 nM). Cell samples were subjected to immunoblot analysis using the indicated antibodies (two are shown in A and the others are not shown). Immunoblot analysis confirmed the inhibition of the mTOR signaling pathway. B, LC3 quantitative analysis of the immunoblot data in cell samples using densitometry scanning. The LC3-II/LC3-I ratio was calculated. Data are expressed as mean± S.E. ($n=6$). **, $P<0.01$ *versus* the control.

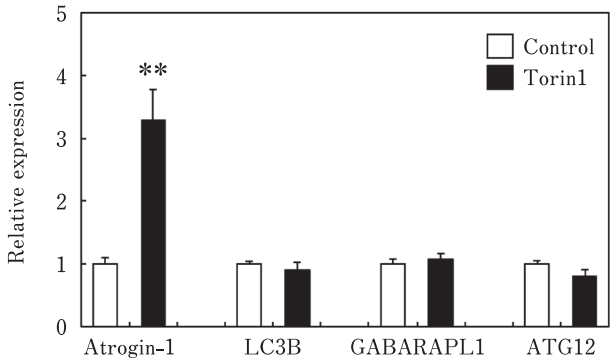

Fig. 2. **Effects of Torin1 on the expression of atrogin-1/MAFbx and autophagy-related genes (LC3B, GABARAPL1, and ATG12) in chick myotubes.** Cells were incubated for 3 h in serum-free M-199 medium with Torin1 (100 nM). Data are expressed as mean±S.E. (n=6). **, $P<0.01$ *versus* the control.

ribosomal protein (Ruvinsky and Meyuhas, 2006) to stimulate mRNA translation (Thoreen *et al*., 2012). Torin1 is an ATP-competitive inhibitor of mTOR that inactivates mTORC1 and mTORC2. Rapamycin is also an inhibitor of mTORC1 but not mTORC2. Torin1 is much more effective than rapamycin in influencing the two processes because rapamycin inhibits mTORC1 incompletely (Thoreen *et al*., 2009). In the present study, we used Torin1 but not rapamycin as an inhibitor of mTOR. Torin1 inhibited the phosphorylation of AKT, S6K1, S6 ribosomal protein, and 4E-BP1 in chick myotubes, indicating that Torin1 completely inhibits mTOR activity in chick myotubes.

The effects of Torin1 on autophagy are shown in Fig. 1B. The LC3-II/LC3-I ratio increased significantly ($P<0.01$) in chick myotubes incubated with Torin1 (100 nM) for 3 h, indicating that Torin1 stimulated autophagy in chick skeletal muscle cells. Autophagy plays an important role under catabolic conditions, during which protein aggregates and organelles are degraded via the formation of autophagosomes, followed by the fusion of lysosomes (Mizushima, 2007; Tooze and Yoshimori, 2010). During autophagosome formation, LC3 is lipidated, converting LC3-I to LC3-II (lipidation) (Kabeya *et al*., 2000). Therefore, the LC3-II/LC3-I ratio is an index of the degree of autophagy. Measuring the conversion of LC3-I to LC3-II using western blotting is considered a reliable method for determining autophagosome formation (Mizushima *et al*., 2010; Naito *et al*., 2013). In this study, inhibition of mTOR induced autophagy in chick myotubes.

We also examined the effect of Torin1 on the expression of autophagy-related genes (LC3B, GABARAPL1, and ATG 12) and atrogin-1/MAFbx in chick myotubes (Fig. 2). LC 3B, GABARAPL1, and ATG12 mRNA levels were not affected by Torin1, indicating that mTOR inhibition does not affect autophagy at a transcriptional level in chick myotubes. Atrogin-1/MAFbx mRNA expression was increased significantly ($P<0.01$) by Torin1, indicating that mTOR inhibition stimulates the ubiquitin-proteasome proteolytic pathways in chick myotubes. Previous studies have shown that atrogin-1/MAFbx expression is regulated by the mTOR signaling pathway in murine C2C12 myotubes (Latres *et al*., 2005; Herningtyas *et al*., 2008). In the present study, atrogin-1/MAFbx expression was up-regulated by Torin1 (mTOR inhibition), indicating that its expression in chick myotubes is also regulated by the mTOR signaling pathway. Atrogin-1/MAFbx, a muscle-specific ubiquitin ligase, is highly expressed in skeletal muscles undergoing atrophy, and its expression is associated with the rate of muscle protein degradation and in turn, muscle size. Atrogin-1/MAFbx plays a critical role in muscle proteolysis, and the level of atrogin-1/MAFbx gene expression is a reliable measure of muscle proteolysis (Ohtsuka *et al*., 2011). Our results revealed that the mTOR signaling pathway controls protein degradation through autophagy and regulates the ubiquitin-proteasome pathway in chick myotubes. Recently, Zhao *et al*. (2015) demonstrated that mTOR inhibition activates protein degradation through the ubiquitin-proteasome proteolytic pathway and autophagy in murine C2C12 myotubes. These results are consistent with our findings. The effect of mTOR inhibition on autophagy and the ubiquitin-proteasome proteolytic pathway in chicken skeletal muscle cells has not been previously reported. To the best of our knowledge, this study is the first to demonstrate that mTOR inhibition stimulates autophagy and the ubiquitin-proteasome proteolytic pathway in chicken skeletal muscle cells.

The effects of fasting on mTOR signaling pathway in chick skeletal muscle are illustrated in Fig. 3A. We used fasting as an approached for mTOR inhibition in skeletal muscle since it is an established condition that inhibits phosphorylation of mTOR target proteins (Mammucari *et al*., 2007; Naito *et al*., 2013; Shavlakadze *et al*., 2013; Saneyasu *et al*., 2017). The phosphorylation of AKT (Ser473), p70 ribosomal S6 kinase 1 (S6K1, Thr389), S6 ribosomal protein (Ser235/236), and 4E-BP1(Thr37/46) was inhibited by fasting, thereby that fasting inhibited AKT and mTOR signaling (phosphorylation of S6K1, S6 ribosomal protein, and 4E-BP1). Immunoblot analysis confirmed the inhibition of the mTOR signaling pathway in chicken skeletal muscle. Activation of mTOR signaling downstream of AKT stimulates protein synthesis and inhibits protein degradation by phosphorylating two major targets, S6K1 and 4E-BP1. Fasting inhibits the phosphorylation of AKT in mouse and chicken skeletal muscles (Mammucari *et al*., 2007; Shavlakadze *et al*., 2013; Saneyasu *et al*., 2017). In addition, fasting inhibits mTOR activity (phosphorylation of S6K1and 4E-BP1) in mouse skeletal muscles (Mammucari *et al*., 2007). Fasting also decreases the phosphorylation of S6 ribosomal protein downstream of S6K1, a maker of mTOR activity, in skeletal muscles (Shavlakadze *et al*., 2013; Saneyasu *et al*., 2017). In this study, fasting also inhibited AKT and mTOR signaling in chick skeletal muscle.

The effects of fasting on the LC3-II/LC3-I ratio in chick skeletal muscle are illustrated in Fig. 3B. Fasting for 24 h significantly ($P<0.01$) increased the LC3-II/LC3-I ratio, in-

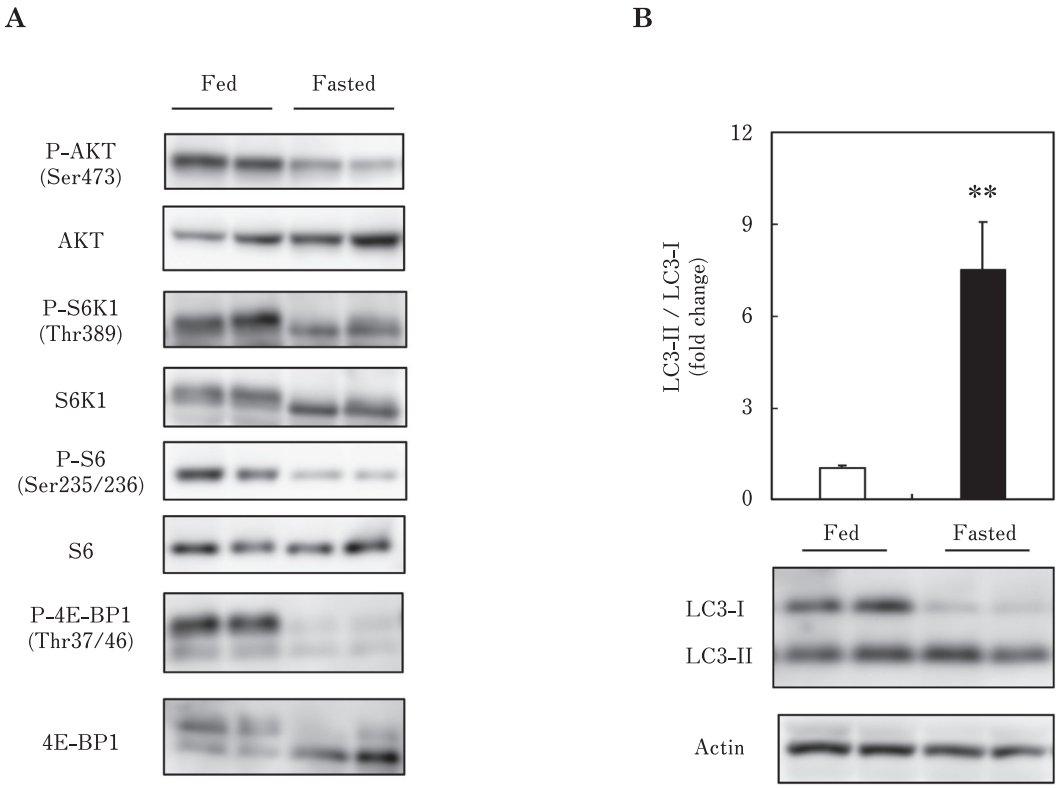

Fig. 3. **Effect of fasting on AKT and mTOR signaling and autophagy in chick pectoralis muscle.** A, chicks were fasted for 24 h. Muscle samples were subjected to immunoblot analysis using the indicated antibodies (two are shown in A and the others are not shown). Immunoblot analysis confirmed the inhibition of the mTOR signaling pathway. B, LC3 quantitative analysis of the immunoblot data in cell samples using densitometry scanning and the resulting LC3-II/LC3-I ratio. Data are expressed as mean±S.E. ($n=6$). **, values are significantly different at $P<0.01$.

dicating that fasting stimulates autophagy in chicken skeletal muscle. Saneyasu *et al*. (2015) reported that fasting for 24 h induced autophagy (LC3 lipidation) in layer and broiler skeletal muscles. This is consistent with the findings of our study. We also examined the effect of fasting on the expression of autophagy-related genes (LC3B, GABARAPL1 and ATG12) and atrogin-1/MAFbx in chick skeletal muscle (Fig. 4). Fasting significantly ($P<0.01$) increased GABARAPL1 mRNA levels, and decreased the levels of LC3B and ATG12 mRNA.

Autophagy, an intracellular bulk protein degradation system, is enhanced under different catabolic conditions such as fasting, and is largely regulated by post-translational lipid modification of the LC3 protein (Kabeya *et al*., 2000; Naito *et al*., 2013). LC3 lipidation is widely used as a measure of autophagy (Mizushima *et al*., 2010; Naito *et al*., 2013). Recently, several studies have demonstrated that autophagy is involved in the transcriptional regulation of autophagy-related genes in the skeletal muscle (Mammucari *et al*., 2007; Zhao *et al*., 2007; Seiliez *et al*., 2010). Fasting also increases GABARAPL1 mRNA expression in chicken skeletal muscles. Consistent with our results, fasting for 1 day has been previously shown to stimulate the expression of GABARAPL1 mRNA in rodent skeletal muscle (Mammucari *et al*., 2007). In addition, fasting for 1 day increases the expression of LC3B mRNA but not ATG12 in rodent skeletal muscle (Mammucari *et al*., 2007). Notably, fasting decreases both LC3B and ATG12 mRNA levels in chick skeletal muscle (pectoralis muscle). We examined LC3B mRNA expression in various skeletal muscles including the iliotibialis lateralis, gastrocnemius lateralis, and flexor perforans et perforatus digiti II muscles of chicks. While fasting induced a significant 3-fold increase in LC3B mRNA expression in the flexor perforans et perforatus digiti II muscle, these was no increase in LC3B mRNA expression in the iliotibialis lateralis and gastrocnemius lateralis muscles of chicks (data not shown). We therefore hypothesize that fasting differentially regulates the expression of autophagy-related genes in various chicken skeletal muscles. During fasting, the expression of atrogin-1/MAFbx increases before

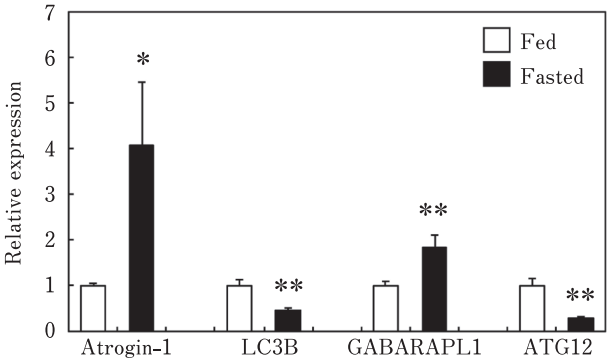

Fig. 4. **Effect of fasting on the mRNA expression of atrogin-1/MAFbx and autophagy-related genes (LC3B, GABARAPL1, and ATG12) in chick pectoralis muscle.** Chicks were fasted for 24 h. Data are expressed as mean± S.E. (n=5–6). Values are significantly different at * $P<0.05$; ** $P<0.01$.

mouse skeletal muscle weight loss and its mRNA expression level is also high during periods of rapid protein degradation (Gomes *et al*., 2001). In chicken skeletal muscles, fasting increases atrogin-1/MAFbx mRNA expression (Li *et al*., 2011; Nakashima and Ishida, 2015; Saneyasu *et al*., 2015). In this study, fasting significantly ($P<0.05$) increased atrogin-1/MAFbx mRNA expression (Fig. 4), indicating that fasting stimulates the ubiquitin-proteasome proteolytic pathway and autophagy in chick skeletal muscle via the mTOR signaling pathway.

In this study, mTOR inhibition was achieved by treatment with inhibitor (Torin1) *in vitro* in chick myotubes and fasting *in vivo* in chicken skeletal muscle. Both treatments inhibited mTOR activity completely, and induced autophagy and the ubiquitin-proteasome proteolytic pathway in chicken skeletal muscle. Growth factors (insulin and insulin-like growth factor-I), nutrients (amino acids), and cellular energy levels all influence skeletal muscle mass and mTOR activity in the skeletal muscle (Miyazaki and Esser, 2009). Conversely, lack of nutrients or growth factors and low energy levels in the skeletal muscle induces autophagy (Sandri, 2008). We have previously reported that insulin, insulin-like growth factor-I, and amino acids suppress autophagy in chick myotubes (Nakashima and Ishida, 2018). This study showed that inhibition of mTOR induces autophagy in chick skeletal muscle. Our findings indicate that growth factors, nutrients, and cellular energy levels control autophagy and mTOR signaling, in addition to influencing chicken skeletal muscle mass. Further investigation is needed on the effects of growth factors, nutrients, and cellular energy levels on autophagy and the mTOR signaling pathway in chicken skeletal muscles.

This study demonstrated that inhibition of mTOR stimulates the autophagy-lysosome as well as the ubiquitin-proteasome proteolytic pathways in chicken skeletal muscle.

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