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Regulation of autophagy in chick myotube cultures: Effect of uncoupling mitochondrial oxidative phosphorylation

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Abstracts: Skeletal muscles have a high demand for ATP, which is met largely through mitochondria oxidative phosphorylation. Autophagy is essential for the maintenance of skeletal muscle mass under catabolic conditions. This study investigated the effect of uncoupling mitochondrial oxidative phosphorylation on autophagy in chicken skeletal muscle. Chick myotubes were incubated with the mitochondrial uncoupler carbonyl cyanide m-chlorophenyl hydrazone (CCCP) at 25 μM for 3h. CCCP prevented the phosphorylation of p70 ribosomal S6 kinase 1 (Thr389), S6 ribosomal protein (Ser240/244), and eukaryotic translation initiation factor 4E-binding protein 1 (Thr37/46), which are the measures of the mechanistic target of rapamycin complex 1 (mTORC1) activity. CCCP significantly increased cytoplasmic and mitochondrial LC3-II content, which act as indices of index for autophagosome formation and mitophagy, respectively, but did not influence the expression of autophagy-related genes LC3B, GABARAPL1, and ATG12. Finally, surface sensing of translation method revealed that protein synthesis, a highly energy consuming process, was significantly decreased upon CCCP treatment. These results indicate that the uncoupling of mitochondrial oxidative phosphorylation stimulates autophagy and inhibits protein synthesis through mTORC1 signaling in chick myotube cultures.

Key words: autophagy, carbonyl cyanide m-chlorophenyl hydrazone (CCCP), chick myotubes, mitophagy, protein synthesis

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Introduction

Macroautophagy, hereafter referred to as autophagy, is a cellular lysosomal protein degradation pathway and 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 they are converted into autolysosomes the action of lysosomal hydrolases[1,2]. Autophagy is induced under energy and nutrients deficiency to provide amino acids for protein synthesis, energy production, and gluconeogenesis, under physiological and nutritional conditions[3,4].

Skeletal muscles mass is determined by the difference between the rates of protein synthesis and degradation. Animal experiments have consistently demonstrated that protein degradation by autophagy increases the number of skeletal muscles undergoing atrophy[5,6]. In addition, autophagy is essential for the maintenance of skeletal muscle mass under catabolic conditions[7,8]. Notably, the stimulation of autophagy in skeletal muscles can induce the production of amino acids, thereby increasing intracellular ATP levels[3].

Mitochondria are the main source of ATP, whose production via oxidative phosphorylation relies on a proton electrochemical gradient. Skeletal muscle serves as a source of amino acids for ATP production under catabolic conditions. Carbonyl cyanide m-chlorophenyl hydrazone (CCCP) is a protonophore that renders the mitochondrial inner membrane permeable to protons, thereby dissipating the proton gradient across the membrane[9]. The latter causes mitochondrial uncoupling, whereby the transfer of electrons through the electron transport chain is no longer coupled to ATP production[9]. At present, it remains unclear if uncoupling of mitochondrial oxidative phosphorylation in skeletal muscle triggers autophagy.

The mechanistic target of rapamycin (mTOR), a serine/threonine kinase, is a regulates cellular processes, such as growth, proliferation, differentiation, survival, and metabolism[10,11]. mTOR complex 1 (mTORC1) is a central regulator of protein metabolism[12]. It stimulates RNA translation by phosphorylating downstream target proteins, such as p70 ribosomal S6 kinase

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1 (S6K1) and eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1)[13]. mTORC1 controls cell growth by regulating protein synthesis machinery, autophagy, and the ubiquitin–proteasome proteolytic pathway[14,15]. Crucially, mTOR signaling regulates autophagy in skeletal muscles[16,17], although the underlying mechanism in chicken is not fully understood.

Protein synthesis in skeletal muscle is considered as an energy-consuming cellular process. We also focused on the effect of uncoupling of mitochondrial oxidative phosphorylation on protein synthesis in chick myotubes. The surface sensing of translation (SUnSET) method was developed and validated against radioactive-based methods for measuring changes in protein synthesis in cultured cells[18]. Consequently, in the current investigation, we utilized the SUnSET method to examine the effect of CCCP on protein synthesis in chick myotubes. We then applied the method to show that CCCP repressed protein synthesis in chick myotube cultures.

Therefore, this study aimed to investigate the effect of CCCP, uncoupler of oxidative phosphorylation in mitochondria, on autophagy and mTOR signaling in chick myotube cultures. It also demonstrated that the uncoupling of oxidative phosphorylation in mitochondria promotes autophagy by inhibiting mTOR signaling in chicken skeletal muscle cultures.

Materials and Methods

Antibodies

Antibodies against microtubule-associated protein 1 light chain 3B (LC3B, #2775), S6K1 (#9202), phospho-S6K1 (Thr389, #9205), S6 ribosomal protein (#2217), phospho-S6 ribosomal protein (Ser240/244, #2215), 4E-BP1 (#9452), phospho-4E-BP1 (Thr37/46, #9459), and COX IV (#4850) were purchased from Cell Signaling Technology. Antibodies against actin (A2066) and α-tubulin (T9026) were purchased from Sigma-Aldrich. The antibody against puromycin (clone 12D10; MABE343) was purchased from Merck Millipore.

Cell Culture

Myoblasts were isolated from thigh muscles of 13-day-old chick embryos[19]. Briefly, the muscle tissue obtained from the embryo was digested with dispase (GIBCO), and the cell suspension was transferred to an uncoated culture dish to allow fibroblast attachment. The cells were counted and then plated onto gelatin-coated six-well plates (Iwaki SciTech) 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°C in a humidified atmosphere containing 5% CO_2 for 7 days. On day 7, the cells formed myotubes and were incubated in serum-free M-199 medium containing 25 μM CCCP (Sigma-Aldrich) for 3 h. 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 chick myotubes using TRIzol reagent (Invitrogen) following the manufacturer's instructions. Complementary DNA was synthesized from total RNA using random primers (TaKaRa) and ReverTra Ace (TOYOBO). The primer sequences were as follows: LC3B (NM_001031461), forward: 5′-TCCGAGATCAGCATCCAACT-3′ and reverse: 5′-CACCATGCTGTGTCCGTTC-3′; GABA(A) receptor-associated protein like 1 (GABARAPL1, XM_001231887), forward: 5′-CCGACAGAGTCCCCGTAATT-3′ and reverse: 5′-ATGGTAGCACTTGTGGGAGG-3′; autophagy-related 12 (ATG12, XM_424963), forward: 5′-CGGAAAGGACCCCAGAGAG-3′ and reverse: 5′-CTTGATGAAGTCGCACAGGC-3′; and 18S ribosomal RNA (AF173612), forward: 5′-AAACGGCTACCACATCCAAG-3′ and reverse: 5′-CCTCCAATGGATCCTCGTTA-3′. mRNA levels were measured by real-time PCR using a LightCycler® instrument (Roche Diagnostics) and a QuantiTect SYBR Green PCR system (QIAGEN). Relative expression levels were calculated using the standard curve method and normalized to 18S RNA as an internal control.

Isolation of mitochondria

Mitochondria were isolated from the cultured cells using a commercially available kit (KC010100; BioChain), according to the manufacturer's instructions.

Western Blotting

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

Equal amounts of protein lysates were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto nitrocellulose or polyvinylidene difluoride membranes. 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 membranes were then incubated with a primary antibody overnight at 4°C. After incubation with the primary antibody, the membranes were incubated with a horseradish peroxidase-conjugated secondary antibody (GE HealthCare) for 1 h at room temperature. The bands were visualized using a western blotting detection reagent (GE HealthCare) and the LAS-3000 mini image analyzer (FUJIFILM). Relative band intensity was quantified using ImageJ software (National Institutes of Health). In reprobing immunoblots, membranes were stripped with stripping buffer (100 mM 2-mercaptoethanol, 2% sodium dodecyl sulfate, 62.5 mM Tris–HCl, pH 6.7) for 30 min at 50°C, washed with TBS-T, and then re-blocked prior to incubation with another primary antibody.

Protein synthesis measurement

Protein synthesis in chick myotubes was determined as previously described using the SUnSET method[20], which measures puromycin incorporation into nascent peptide chains[18]. Briefly, puromycin was added to the culture medium (1 μM final concentration) for 30 min prior to cell lysis with RIPA lysis buffer (Santa Cruz Biotechnology). The amount of puromycin incorporated into the newly synthesized protein was determined by western blotting. The membranes were stained with Ponceau S to

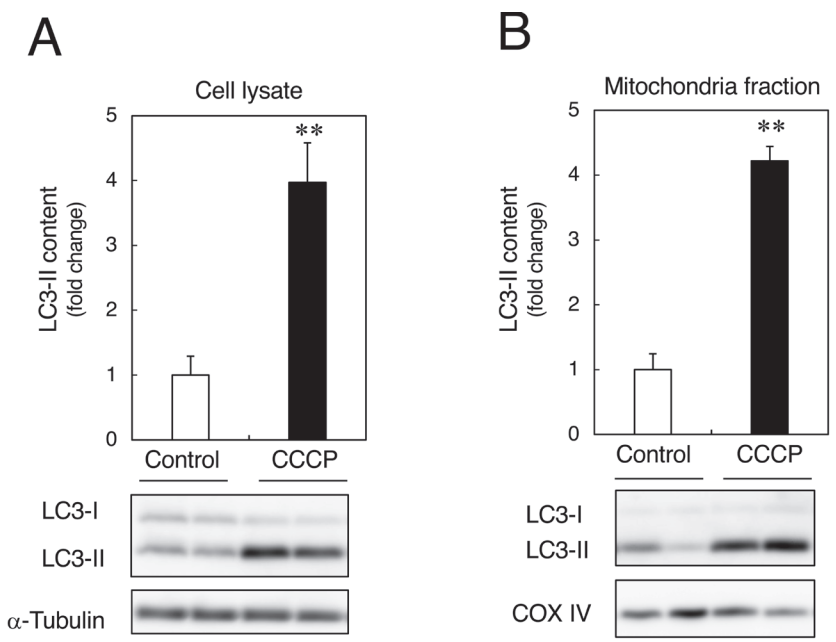

Fig. 1. **Effect of CCCP on autophagy (A) and mitophagy (B) in chick myotubes.** Cells were incubated for 3 h in serum-free M-199 medium containing 25 μM CCCP. LC3 was quantified based on immunoblot data using densitometry scanning and the resulting LC3-II/LC3-I ratio. α-Tubulin and COX IV were used as a loading control. Data are expressed as the mean ± SE (n = 5–6). **P < 0.01 versus the control.

ensure equal protein loading.

Statistical Analysis

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

Results and Discussion

The effect of CCCP on autophagy is shown in Figure 1A. LC3-II content increased significantly (P < 0.01) in chick myotubes incubated with 25 μM CCCP for 3 h, indicating that CCCP stimulated autophagy in chick skeletal muscle cells. The effect of CCCP on mitophagy is shown in Figure 1B. LC3-II content in the mitochondrial fraction also increased significantly (P < 0.01) following incubation with CCCP. These results indicate that uncoupling of oxidative phosphorylation in mitochondria stimulates autophagy and mitophagy in chick myotubes. Autophagy plays an important role during catabolism, where protein aggregates and organelles are degraded via the formation of autophagosomes, followed by the fusion of lysosomes[1,2]. During the formation of autophagosome, LC3 is lipidated, converting LC3-I to LC3-II (lipidation)[21]. Accordingly, LC3-II content is an indicator the degree of autophagy. Western blotting, which can be used to measure the conversion of LC3-I to LC3-II, represents a reliable method for determining autophagosome formation[22,23]. Consistent with the present results, CCCP has been previously shown to stimulate autophagy in murine C2C12 myotubes[24–26]. To the best of our knowledge, the present study is the first to demonstrate that CCCP stimulates autophagy in chicken skeletal muscle cells. Mitophagy is tasked with maintaining homeostasis degrading damaged mitochondria[27]. The mitochondrial LC3-II content, as an index of mitophagy, was also increased following CCCP treatment, indicating that treating chick myotubes with CCCP induced the removal of damaged mitochondria, thus echoing what had been observed in murine C2C12 myotubes and skeletal muscles[24,25].

Mitochondria produce ATP through a complex interconnected metabolic network using multiple energy sources, such as amino acids, lipids, and carbohydrate derivatives. Mitochondrial energy metabolism is highly regulated to constantly meet the energy needs of the cell and utilize available energy substrates[28]. At the same time, autophagy and mitophagy ensure quality control in cells[29]. In this study, the uncoupling of oxidative phosphorylation by CCCP stimulated autophagy and mitophagy in chick myotubes. We therefore examined the effect of CCCP on the expression of autophagy-related genes in chick myotubes (Figure 2). LC3B, GABARAPL1, and ATG12 were not affected by CCCP, indicating that uncoupling of oxidative phosphorylation did not promote autophagy at the transcriptional level. Previously, we reported that hormones and nutrients regulated autophagy at the post-translational level rather than at the transcriptional level in chick skeletal muscle cultures[30]. In chick myotube cultures, autophagy at the post-translational level but not at the tran-

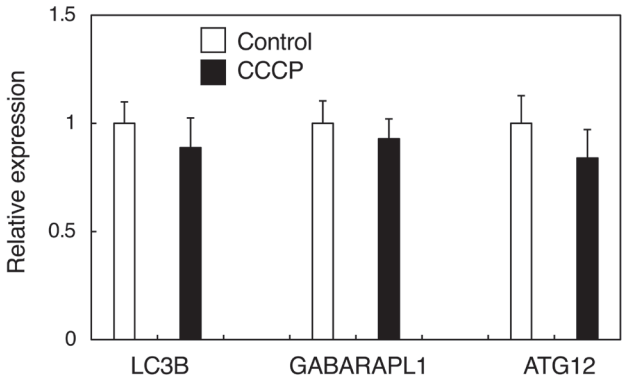

Fig. 2. **Effect of CCCP on the expression of autophagy-related genes (LC3B, GABARAPL1, and ATG12) in chick myotubes.** Cells were incubated for 3 h in serum-free M-199 medium containing 25 μM CCCP. Data are expressed as the mean ± SE (n = 6).

scriptional level may be important in regulating skeletal muscle proteolysis.

The serine/threonine kinase mTOR regulates essential cellular processes such as growth, proliferation, differentiation, survival, and metabolism[10,11]; whereas mTORC1 is the central regulator of protein metabolism[12]. The effect of CCCP on mTORC1 signaling in chick skeletal muscle cells is summarized in Figure 3. Phosphorylation of S6K1 (Thr389), S6 ribosomal protein (Ser240/244), and 4E-BP1(Thr37/46) was prevented by CCCP, indicating that the uncoupling of oxidative phosphorylation inhibited mTORC1 signaling in chick myotubes. mTOR stimulates cell growth through the mTORC1-activated phosphorylation of S6K1 and 4E-BP1[31]. Here, we describe the essential role of mTORC1 signaling in autophagy induction after the uncoupling of oxidative phosphorylation by CCCP, which enables the targeting of uncoupled mitochondria for autophagic degradation[32]. Skeletal muscle is the largest reservoir of amino acids for energy production during catabolism and maintaining homeostasis. Starvation induces autophagy and blocks mTOR signaling in mammalian skeletal muscles[23]. We previously observed that the same trend also in chicken skeletal muscle[17], as well as in chicken myotube cultures treated with mTOR inhibitor[17]. The present study reports the stimulation of autophagy and mitophagy plus inhibition mTOR following uncoupling of oxidative phosphorylation in chick myotubes.

The effect of CCCP on protein synthesis in chick myotubes is shown in Figure 4. Using the SUnSET method, the content of puromycin was found to drop significantly ($P < 0.01$) in response to CCCP treatment, indicating that CCCP inhibited protein synthesis in chick myotube cultures. Using a radioactive method, McLeod *et al*.[33] reported that CCCP inhibited protein synthesis in rat ventricular cardiomyocytes. The present study is the first to use the SUnSET method to show that mitochondrial uncoupling of oxidative phosphorylation suppresses protein syn-

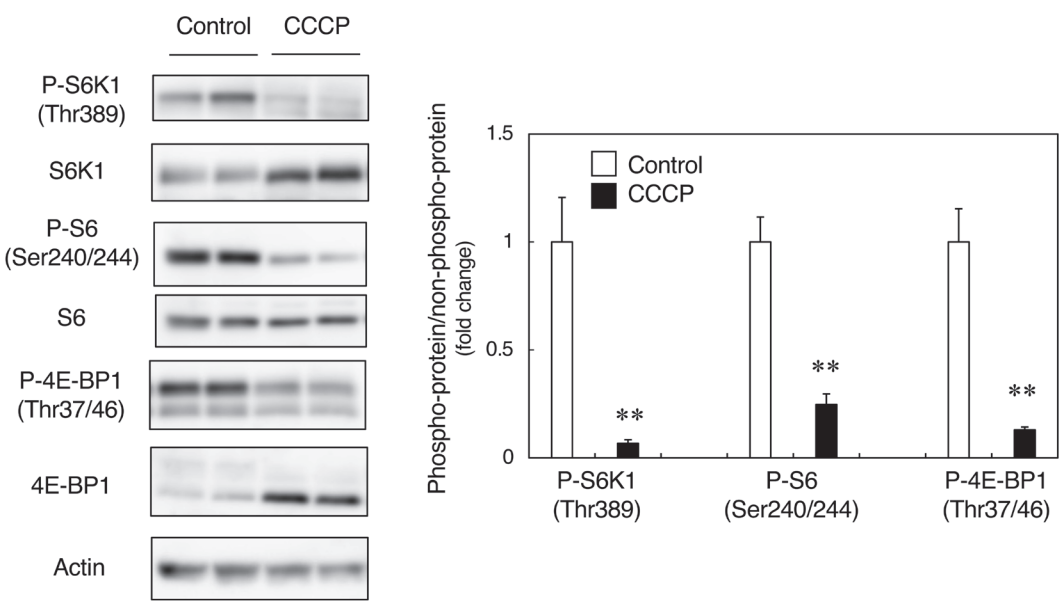

Fig. 3. **Effect of CCCP on mTOR signaling in chick myotubes.** Cells were incubated for 3 h in serum-free M-199 medium containing 25 μM CCCP. Cell samples were subjected to immunoblot analysis using the indicated antibodies. Actin was used as a loading control. Protein phosphorylation and quantification of western blot data were performed using densitometry scanning. Data are expressed as the mean ± SE (n = 6). **P < 0.01 versus the control.

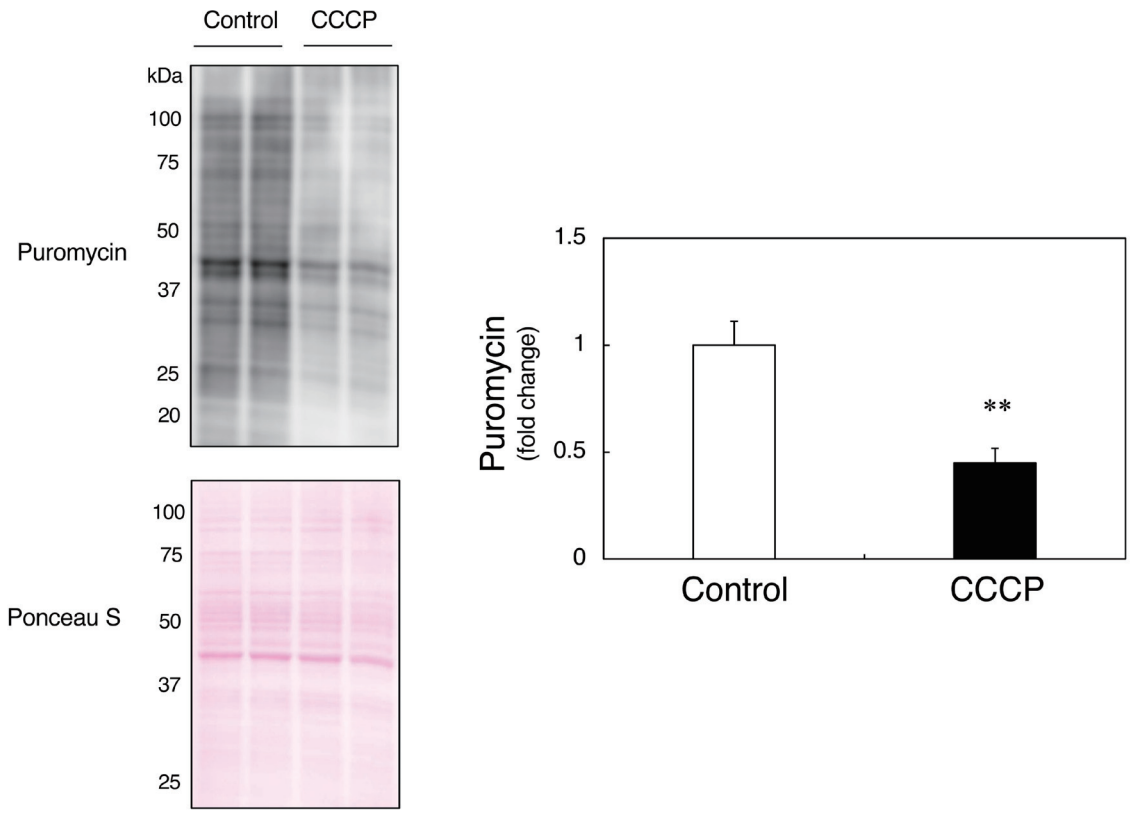

Fig. 4. **Effect of CCCP on protein synthesis in chick myotube cultures.** Cells were incubated for 3 h in serum-free M-199 medium containing 25 μM CCCP. Cell samples were analyzed by western blot using an anti-puromycin antibody. A representative image of the blot stained with Ponceau S to verify equal loading is shown. Puromycin was quantified using densitometry scanning. Data are expressed as the mean ± SE (n = 6). **P < 0.01 versus the control.

thesis in skeletal muscle. Protein synthesis is regulated by the phosphorylation and dephosphorylation of protein factors, such as 4E-BP1 and S6K1, involved initiation and elongation steps, through the activation of the mTORC1 signaling pathway[34]. mTORC1 is an energy and nutrient sensor that activated in response to amino acids, growth factors, and hormones. It is also a positive regulator of anabolism. It promotes protein synthesis through the phosphorylation of 4E-BP1 and S6K1 that phosphorylate S6 ribosomal protein. In our previous study, intracellular energy deprivation mimicked by AMP-activated protein kinase activation inhibited protein synthesis and mTORC1 signaling in chick myotubes[20]. Overall. these studies indicate that ATP generated by the mitochondria is necessary for protein synthesis in chicken skeletal muscles.

Domestic animals are often subjected to stress arising from restraint, fasting, transport, and exposure to high and low environmental temperatures. For example, heat stress induces the formation of reactive oxygen species (ROS) by uncoupling oxidative phosphorylation in the mitochondria of chicken skeletal muscle[35]. Enhanced ROS production triggers oxidative damage on proteins, which can hamper their biological function and enhance susceptibility to proteolysis, resulting in loss of skeletal muscle mass[35]. ROS promote autophagy and mitophagy in skeletal muscles[36], and their production is induced by CCCP[37]. The present findings suggest that autophagy and mitophagy might be regulated via mTOR signaling, as well as in response to reduced protein synthesis following uncoupling of oxidative phosphorylation. Further studies are required to elucidate the impact of stress on autophagy and mTOR signaling in chicken skeletal muscles. In the future, nutrients and antioxidants that influence mTOR signaling could be utilized to regulate avian muscle growth in poultry production.

This study demonstrates that the uncoupling of mitochondrial oxidative phosphorylation by CCCP in chick myotube cultures stimulates autophagy and inhibits protein synthesis through mTORC1 signaling.

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Author Contribution

All authors contributed the conception and design of this study. The preparation of biological samples and data collection were performed by Kazuki Nakashima. Data analysis and interpretation were done by all authors. The first draft of the manuscript was written by Kazuki Nakashima, and all authors commented on previous versions of the manuscript. All authors have read and approved the final version of the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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