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Effects of Insulin-Like Growth Factor-I on the Expression of Atrogin-1/MAFbx in Chick Myotube Cultures

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The expression of atrogin-1/MAFbx, a muscle-specific E3 ubiquitin ligase, is increased in catabolic conditions that result in muscle atrophy. The expression of atrogin-1/MAFbx mRNA is also decreased by the insulin-like growth factor-I (IGF-I) in mammalian skeletal muscle cell cultures. This study investigated the effect of IGF-I on the expression of atrogin-1/MAFbx in chicken skeletal muscle cell cultures. Chick myotubes were incubated with IGF-I for 1, 6, or 24 h. Protein content was increased by IGF-I (100 ng/m*l*) and incubated for 24 h in chick myotubes. The expression of atrogin-1/MAFbx mRNA decreased in the presence of IGF-I (1, 10, and 100 ng/m*l*) for 6 h in chick myotubes. The expression of the m-calpain large subunit and cathepsin B mRNA was not decreased by IGF-I. Phosphorylation of Akt and FOXO1 increased in the presence of IGF-I (100 ng/m*l*) for 1 h in chick myotubes. These results indicate that IGF-I suppresses atrogin-1/MAFbx mRNA expression by phosphorylation of Akt and FOXO1, resulting in an increase in muscle growth in chick myotube cultures.

Key words: Akt, atrogin-1/MAFbx, chick myotubes, FOXO1, IGF-I

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Introduction

Skeletal muscle mass is determined from the difference between the rates of protein synthesis and protein degradation. Studies of animal experiments have consistently demonstrated that protein degradation by the ubiquitin-proteasome system is increased in muscle undergoing atrophy (Mitch and Goldberg, 1996, Dehoux *et al*., 2003, Lecker *et al*., 2004). Proteolysis in catabolic conditions is also primarily due to the activation of the ubiquitin-proteasome proteolytic pathway (Price *et al*., 1996, Lecker *et al*., 2004), whereby the proteins destined to be degraded are linked to a chain of ubiquitin molecules, which targets them for rapid breakdown by the proteasome (Glickman and Ciechanover, 2002). Evidence suggests that atrogin-1, an E3 ubiquitin ligase also referred to as MAFbx (muscle atrophy F-box), plays a pivotal role in muscle atrophy (Gomes *et al*., 2001, Bodine *et al*., 2001). Its expression is increased in catabolic conditions that result in muscle atrophy (Gomes *et al*., 2001, Bodine *et al*., 2001, Dehoux *et al*., 2003). The factors and mechanisms regulating atrogin-1/MAFbx expression in skeletal muscles are poorly understood.

Insulin-like growth factor-I (IGF-I) plays an important role in growth and continues to have anabolic effects in animals. IGF-I stimulates muscle protein synthesis and hypertrophy via the Akt and transcription factors Forkhead box-O (FOXO) signaling pathway, and activation of this pathway can reduce muscle atrophy (Rommel *et al*., 2001). IGF-I inhibits overall protein breakdown, the degradation of myofibrillar proteins (Gulve and Dice, 1989, Vandenburgh *et al*., 1991, Sacheck *et al*., 2004), and the expression of atrogin-1/MAFbx and MuRF1 (Sacheck *et al*., 2004, Stitt *et al*., 2004). Although it is well known that IGF-I has a regulatory effect on the expression of atrogin-1/MAFbx and Akt and the FOXO signaling pathway in mammalian skeletal muscle, such effects in chicken muscle are largely unknown.

Like other tissues, skeletal muscle contains multiple proteolytic systems, each of which may play an important role in muscle atrophy. Intracellular proteolysis is carried out by lysosomal and non-lysosomal pathways in which intracellular proteases are directly responsible for the degradation of proteins. Calpains, i.e., cysteine proteases in the cytosol, are thought to be the main agents of non-lysosomal Ca^{2+}-dependent proteolysis, which occurs within the myofibril, and are capable of carrying out the initial step in myofibrillar

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proteolysis (Goll *et al*., 1991, Goll *et al*., 1992). It is well established that cathepsins, i.e. the main agents of lysosomal degradation, contribute to muscle protein breakdown (Hall-Angeras *et al*., 1991). Lysosomal proteases degrade sarcoplasmic proteins and release myofibrillar proteins (Lowell *et al*., 1986). Although the effect of IGF-I on all these protein degradation systems in mammalian skeletal muscle has been well studied, its effect remains unknown in chicken muscle.

Thus, in this study, the effect of IGF-I on the expression of atrogin-1/MAFbx and Akt and the FOXO signaling pathway in chick myotubes is investigated. We demonstrate that IGF-I suppresses atrogin-1/MAFbx mRNA expression by phosphorylation of Akt and FOXO1, resulting in an increase in muscle growth in chick myotube cultures.

Materials and Methods

Cell Culture

Myoblasts were isolated from the thigh muscles of 13-day-old chick embryos (Nakashima *et al*., 2005). Briefly, the muscle tissue obtained from the embryo was digested with dispase (GIBCO, NY, 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 six-well plates (Iwaki SciTech, Tokyo, Japan). Chick myoblasts were cultured in an M-199 medium containing 15% calf serum and 2.5% chicken embryo extract, and were grown at 37℃ in a humidified 5% CO_2 incubator during a 7 day incubation period. The cells formed myotubes on the seventh day. The myotubes were incubated for 1, 6 or 24 h in a serum-free M-199 medium containing IGF-I (R^3-IGF-I, Sigma Aldrich, St. Louis, USA). The cell protein concentration was determined by BCA assay (Pierce, Rockford, USA) with bovine serum albumin as the standard.

All experimental procedures were conducted in accordance with the guidelines of the Animal Care and Use Committee of the NARO Institute of Livestock and Grassland Science.

Real-time PCR

Total RNA was extracted from chick myotubes using TRIzol reagent (Invitrogen, Carlsbad, USA) according to the manufacturer's directions. Complementary DNA was synthesized from total RNA using random primer (TaKaRa, Tokyo, Japan) and ReverTra Ace (TOYOBO, Osaka, Japan). The sequences of the primers were as follows: atrogin-1/MAFbx, forward: 5′-CCAACAACCCAGAGACCTGT-3′ and reverse: 5′-GGAGCTTCACACGAACATGA-3′; m-calpain large subunit, forward: 5′-ACATCATCGTGCCCTCTACC-3′ and reverse: 5′-GAGATCTCTGCATCGCTTCC-3′; cathepsin B, forward: 5′-CAAGCTCAACACCACTGGAA-3′ and reverse: 5′-TCAAAGGTATCCGGCAAATC-3′; and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), forward: 5′-CCTCTCTGGCAAAGTCCAAG-3′ and reverse: 5′-CATCTGCCCATTTGATGTTG-3′. Levels of mRNA were measured by real-time PCR analysis using a LightCycler® instrument (Roche Diagnostics, Mannheim, Germany) and the QuantiTect SYBR Green PCR system (Qiagen, Tokyo, Japan). The level of GAPDH was measured as an internal control.

Western Blotting

The cells were washed twice with ice-cold PBS and lysed in RIPA buffer (Santa Cruz Biotechnology, CA, USA). The lysate was centrifuged at 14,000 rpm for 5 min at 4℃ and the supernatant was collected. Total protein concentration was estimated by a protein-dye-binding assay using a commercial kit (Bio-Rad, CA, USA) with bovine serum albumin as the standard.

Western blot analysis was performed as described previously (Shimamoto *et al*., 2016). In brief, the samples were electrophoresed on an SDS/10% (w/v) polyacrylamide gel, and then, transferred to a polyvinylidene difluoride membrane (Immobilon-P, Merck Millipoire, MA, USA). The membrane was blocked with 5% (w/v) skim milk in Tris-buffered saline containing 0.1% (v/v) Tween-20 (TBST) for 1 h at room temperature. Subsequently, the blocked membrane was incubated with a primary antibody against Akt (#9272; Cell Signaling Technology, MA, USA), phospho-Akt (Ser473) (#9271; Cell Signaling Technology, MA, USA), FOXO1 (#9454; Cell Signaling Technology, MA, USA), and phospho-FOXO1 (Ser256) (#9461; Cell Signaling Technology, MA, USA) in Can Get Signal I (TOYOBO, Osaka, Japan) overnight at 4℃ (1:5000 dilution). Then, these membranes were incubated with a secondary antibody against rabbit IgG (sc-2030; Santa Cruz Biotechnology, CA, USA) in Can Get Signal II (TOYOBO, Osaka, Japan) at 37℃ for 2 h (1:5000 dilution). Because there were no significant differences in the levels of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) protein between two treatments, the level of GAPDH was used as an internal standard. All membranes were incubated with a primary antibody against GAPDH (sc-20357, Santa Cruz Biotechnology, CA, USA) in Can Get Signal I overnight at 4℃ (1:5000 dilution). Then, these membranes were incubated with a secondary antibody against goat IgG (sc-2020, Santa Cruz Biotechnology, CA, USA) in Can Get Signal II at 37℃ for 2 h (1:5000 dilution). The blots were detected using EzWestLumi plus and Ez-Capture MG (ATTO Corporation, Tokyo, Japan) according to the manufacturer's instructions. Relative band intensity was quantified using ImageJ software (National Institutes of Health, MA, USA).

Statistical Analysis

Data were analyzed by Student's *t*-test or one-way analysis of variance followed by Tukey's multiple-range test for *post hoc* analyses. A P value <0.05 was considered to be statistically significant. Data were expressed as means±standard deviation (SD).

Results and Discussion

This study investigates the effect of IGF-I on the expression of atrogin-1/MAFbx mRNA in chick myotube cultures. The effects of IGF-I on protein content are shown in Fig. 1A. The cell protein content was significantly increased by IGF-I (100 ng/m*l*) in chick myotube cultures. This result indicates that IGF-I stimulates muscle growth in chick skeletal muscle

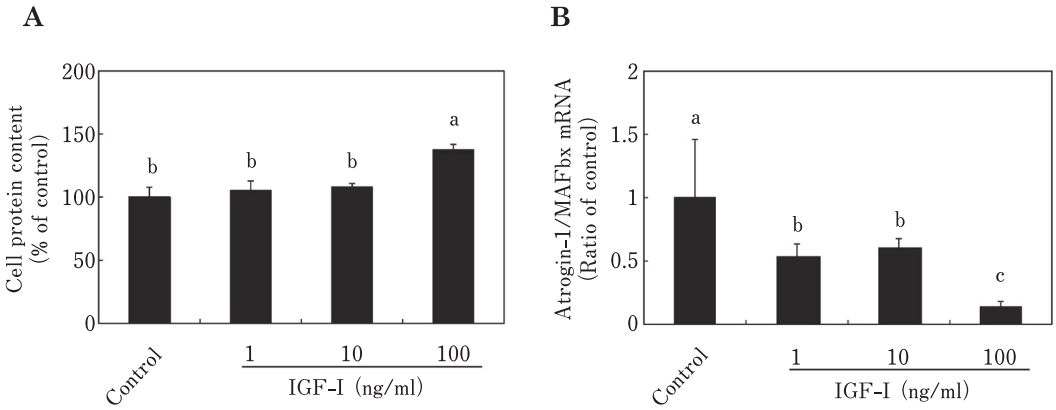

Fig. 1. **Effect of IGF-I on cell protein content (A) and expression of atrogin-1/MAFbx mRNA (B) in chick myotube cultures.** Cells were incubated for 6 or 24 h in serum-free m-199 medium with IGF-I (1, 10, and 100 ng/m*l*). Data are expressed as means±SD ($n=6$) .Values that do not share the same superscript letter are significantly different ($p<0.05$).

cells. It is well known that IGF-I promotes muscle growth in mammalian cultured skeletal muscle cells (Rommel *et al*., 2001, Sacheck *et al*., 2004). These results are consistent with ours. In this study, IGF-I stimulates muscle growth in chick myotube cultures. Muscle growth is thought to be controlled by a dynamic balance between protein synthesis and degradation.

Atrogin-1/MAFbx, a muscle-specific ubiquitin ligase, is highly expressed in skeletal muscle undergoing atrophy, and its expression is related to the rate of muscle protein degradation, and thus, muscle size. Atrogin-1/MAFbx plays a critical role in muscle proteolysis, and the level of atrogin-1/MAFbx gene expression is a reliable index of muscle proteolysis in chicken (Ohtsuka *et al*., 2011). Although it is well known that IGF-I have a regulatory effect on the expression of atrogin-1/MAFbx in mammalian skeletal muscle, its effect in chicken muscle cells is largely unknown. Atrogin-1/MAFbx mRNA expression is shown in Fig. 1B. Atrogin-1/MAFbx mRNA expression was significantly reduced in the presence of IGF-I (Fig. 1B, 1, 10, and 100 ng/m*l*) in chick myotube cultures. This result confirms the effects IGF-I as a regulator of atrogin-1/MAFbx in cultured chick skeletal muscle cells. IGF-I stimulates muscle growth by reducing muscle atrophy (Sacheck *et al*., 2004). IGF-I has also been reported to reduce protein degradation in myotubes (Hong and Forsberg, 1994, Sacheck *et al*., 2004), and this effect has been partly attributed to the suppression of atrogin-1/MAFbx and MuRF1 (Sacheck *et al*., 2004, Stitt *et al*., 2004). In this study, atrogin-1/MAFbx expression was suppressed by IGF-I in chick myotube cultures. It has been reported that insulin suppresses atrogin-1/MAFbx expression in quail QT6 fibroblasts (Tesseraud *et al*., 2007) and chicken skeletal muscles (Dupont *et al*., 2008). However, the effect of IGF-I on atrogin-1/MAFbx in chicken skeletal muscle has not been reported. Our data are supported by these previous studies and are extend by showing that the expression of atrogin-1/MAFbx is also regulated by IGF-I in chick skeletal muscle cells.

Multiple proteolytic systems play a major role in various situations of protein loss and muscle wasting. The intercellular proteolytic processes found in skeletal muscle include various proteases such as lysosomal acidic cathepsins (Hall-Angeras *et al*., 1991) and Ca^{2+}-dependent calpains (Goll *et al*., 1991, Goll *et al*., 1992). Protein can also be degraded by the ATP-dependent ubiquitin-proteasome system (Coux *et al*., 1996). Ubiquitin-proteasome constitutes an essential pathway for accelerated proteolysis in various animal models of muscle wasting (Mitch and Goldberg, 1996, Lecker *et al*., 1999). However, despite the presence of multiple pathways of degradation, the effect of IGF-I on the expression of these proteolytic systems in chick myotube cultures has been reported. The effect of IGF-I on the expression of other proteolytic-related genes in chick myotube cultures was also examined. The expression of the m-calpain large subunit and cathepsin B mRNA was not affected by it (Fig. 2A and B). These results indicate that IGF-I has no effect on the expression of m-calpain large subunit and cathepsin B mRNA in chick myotube cultures. Hong and Forsberg (Hong and Forsberg, 1994) reported that IGF-I reduces protein degradation but not the expression of m-calpain and cathepsin B in murine L8 myotubes. These results are consistent with ours. Our results show that IGF-I has no effect on the expression of m-calpain and cathepsin B in chick myotubes as well as mammalian myotubes.

The results of Akt and FOXO1 phosphorylation in chick myotubes are shown in Fig. 3. Phosphorylation of Akt was significantly increased by IGF-I (100 ng/m*l*, Fig. 3A). Phosphorylation of FOXO1 was also significantly increased by IGF-I (100 ng/m*l*, Fig. 3B). These results indicate that IGF-I stimulates the phosphorylation of Akt and FOXO1 in the

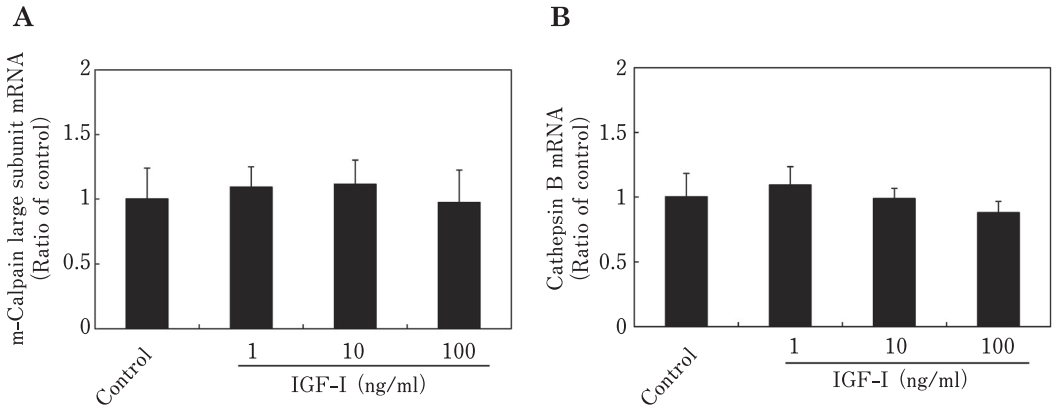

Fig. 2. **Effect of IGF-I on the expression of m-calpain large subunit (A) and cathepsin B (B) mRNA in chick myotube cultures.** Cells were incubated for 6 h in serum-free m-199 medium with IGF-I (1, 10, and 100 ng/m*l*). Results are expressed as ratio of control and are means ±SD ($n=6$) .Values that do not share the same superscript letter are significantly different ($p<0.05$).

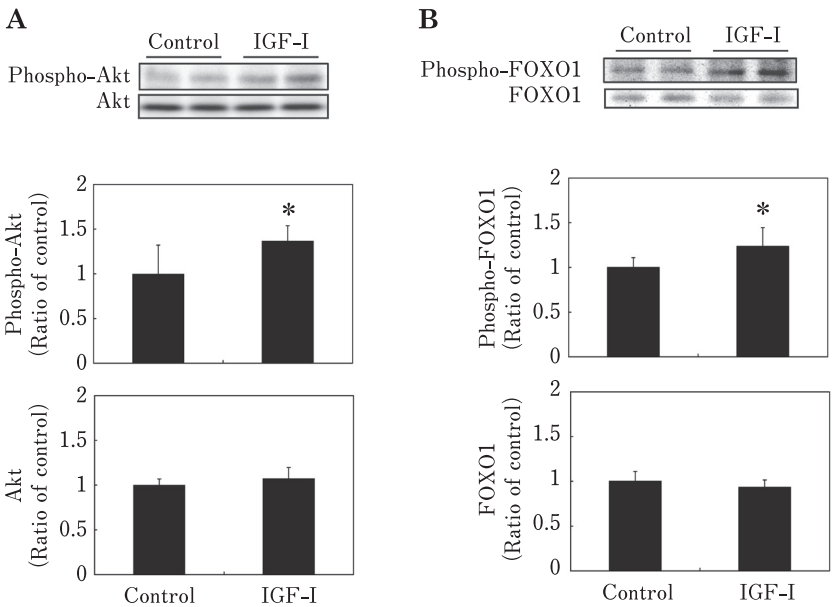

Fig. 3. **Effect of IGF-I on the phosphorylation of Akt (A) and FOXO1 (B) in chick myotube cultures.** Cells were incubated for 1 h in serum-free m-199 medium with IGF-I (100 ng/m*l*). Data are expressed as means ±SD ($n=6$). *$p<0.05$ versus control.

skeletal muscle of chicks. The FOXO subfamily of transcription factors is a downstream target of Akt. This subfamily of consists of three members, FOXO1, FOXO3a, and FOXO4, which are all inactivated by Akt (Daitoku *et al*., 2011). Recent studies have shown that Akt can dominantly inhibit the induction of the atrophy gene of atrogin-1/MAFbx by phosphorylating, thereby inhibiting the function of the FOXO transcription factors (Sandri *et al*., 2004, Stitt *et al*., 2004). IGF-I stimulates phosphoinositide 3-kinase and its downstream effector, Akt, which phosphorylates the FOXO protein (Sandri *et al*., 2004, Stitt *et al*., 2004). Phosphorylated FOXO proteins are unable to translocate to the nucleus, where they promote the transcription of the atrogin-1 gene (Sandri *et al*., 2004, Stitt *et al*., 2004). Since FOXO

transcriptional factors can bind directly to the atrogin-1/MAFbx 5′ untranslated region close to both the putative TATA box and the initiation site of transcription (Sandri *et al*., 2004), they have a critical role in the transcriptional regulation of atrogin-1/MAFbx. In C2C12 myotubes, a marked increase in nuclear FOXO1 and FOXO3a protein expression after removal of growth medium for 2 h while addition of IGF-I increased the levels of phosphorylated FOXO1, FOXO3a, and FOXO4, and consequently suppressed atrogin-1mRNA to the level in C2C12 myotubes (Sandri *et al*., 2004). On the other hand, we previously reported that refeeding stimulates the phosphorylation of FOXO1 and inhibits atrogin-1/MAFbx expression in chick skeletal muscle (Nakashima *et al*., 2006). Furthermore, clenbuterol changed the intracellular localization of phosphorylated FOXO1 and reduced atrogin-1/MAFbx expression in chick skeletal muscle (Shimamoto *et al*., 2016). In this study, our results show that IGF-I stimulates the phosphorylation of Akt and FOXO1, resulting in a decrease in the atrogin-1/MAFbx expression in chick myotubes. These facts indicate that FOXO1 are an important transcription factor that regulates the expression of atrogin-1/MAFbx in chick skeletal muscles.

IGF-I suppresses skeletal muscle protein degradation in chicken *in vivo* (Tomas *et al*., 1998) and *in vitro* (Duclos *et al*., 1993). Furthermore, IGF-I reduces atrogin-1/MAFbx expression via Akt-FOXO signaling pathway in mammalian skeletal muscle cells (Sandri *et al*., 2004, Stitt *et al*., 2004). The present study also shows that IGF-I suppresses atrogin-1/MAFbx expression and stimulation of phosphorylation of Akt and FOXO in cultured chicken muscle cells. However, in this study, the concentration of IGF-I in medium (100 ng/m*l*) is higher than plasma IGF-I concentration (5−50 ng/m*l*) of chicken in several physiological conditions such as embryogenesis, postnatal growth, fasting and refeeding (Kita *et al*., 1996, McMurtry *et al*., 1997, Kita *et al*., 1998). Since this study is performed using cultured chicken cells, which do not provide direct information on *in vivo* conditions in chicken skeletal muscles, further investigations with the administration of IGF-I *in vivo* are necessary.

In conclusion, the present study shows that IGF-I suppresses atrogin-1/MAFbx mRNA expression by the phosphorylation of Akt and FOXO1, resulting in an increase in muscle growth in chick myotube cultures.

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