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Prevention of skeletal muscle atrophy *in vitro* using anti-ubiquitination oligopeptide carried by atelocollagen

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ABSTRACT

Skeletal muscle atrophy occurs when the rate of protein degradation exceeds that of protein synthesis in various catabolic conditions, such as fasting, disuse, aging, and chronic diseases. Insulin-like growth factor-1 (IGF-1) signaling stimulates muscle growth and suppresses muscle protein breakdown. In atrophied muscles, ubiquitin ligase, Cbl-b, increases and stimulates the ubiquitination and degradation of IRS-1, an intermediate in IGF-1 signaling pathway, resulting in IGF-1 resistance. In this study, we evaluated the efficacy of atelocollagen (ATCOL)-transported anti-ubiquitination oligopeptide (Cblin: Cbl-b inhibitor) (consisting of tyrosine phosphorylation domain of IRS-1) in starved C2C12 myotubes. The amount of IRS-1 protein was lower in starved versus unstarved myotubes. The Cblin-ATCOL complex inhibited IRS-1 degradation in a concentration-dependent manner. Myotubes incubated with Cblin-ATCOL complex showed significant resistance to starvation-induced atrophy ($p < 0.01$). Furthermore, the Cblin-ATCOL complex significantly inhibited any decrease in Akt phosphorylation ($p < 0.01$) and localization of FOXO3a to the nucleus in starved myotubes. These results suggest that Cblin prevented starvation-induced C2C12 myotube atrophy by maintaining the IGF-1/Akt/FOXO signaling. Therefore, attachment of anti-ubiquitination oligopeptide, Cblin, to ATCOL enhances its delivery to myotubes and could be a potentially effective strategy in the treatment of atrophic myopathies.

1. Introduction

Skeletal muscle atrophy in the presence of catabolic state associated with fasting, disuse, aging, and chronic diseases is due to imbalance between rates of protein degradation and synthesis [24]. Insulin-like growth factor-1 (IGF-1) signaling promotes muscle growth and suppresses muscle protein breakdown through Akt-dependent phosphorylation and cytosolic sequestration of FOXO transcriptional factors in skeletal myocytes, which leads to inhibition of FOXO-dependent gene expression [22,23,25]. In contrast, IGF-1 signaling is impaired in atrophied muscles, with resultant increase in the transactivation of FOXO target genes, such as atrophy-related genes (atrogenes) that encode atrogin-1 and MuRF-1, which are RING-type ubiquitin ligases known to be critical mediators of atrophic myopathies [1,8].

Cbl-b is a RING-type ubiquitin ligase established as a negative regulator of receptor tyrosine kinase signaling in a variety of cells [9,12,27]. In atrophied muscles, Cbl-b increases and interacts preferentially with IRS-1, an intermediate in the IGF-1 signaling pathway, resulting in loss of IRS-1-activated FOXO-dependant induction of atrogin-1 [17]. Furthermore, Cbl-b-deficient mice are resistant to unloading-induced atrophy [17]. These results suggest that inhibition of Cbl-b-mediated IRS-1 ubiquitination could be a useful therapeutic strategy for muscle atrophy. Based on these findings, we developed an anti-ubiquitination oligopeptide (Cblin: Cbl-b inhibitor) that inhibits the interaction between Cbl-b and IRS-1 [17]. The peptide mimetic of tyrosine^{608}-phosphorylated IRS-1 inhibited Cbl-b-mediated IRS-1 ubiquitination and induction of atrogin-1 [17]. However, peptides are rapidly degraded by aminopeptidases in the body, making it difficult to produce long-term therapeutic effects. Therefore, the development of safe and effective delivery system is critical for the treatment of various muscle atrophies with anti-ubiquitination oligopeptides.

Atelocollagen (ATCOL), a pepsin-treated type I collagen lacking telopeptides at the N and C terminals that confer antigenicity, has been developed as a carrier material [[]]. It is reported that ATCOL

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complexed with small interfering (si)RNA is resistant to nucleases and efficiently transfected into cells, thereby allowing long-term gene silencing. Thus, ATCOL-based delivery method could be a reliable approach to achieve the maximal function of siRNA [16]. Based on its practical use as a siRNA delivery, we have developed an ATCOL-based oligonucleotide system for the delivery of a myostatin-targeting siRNA into skeletal muscles and recently reported that local administration of siRNA attached to ATCOL resulted in marked stimulation of muscle growth and recovery of muscle function in mutant caveolin-3 transgenic mice [11]. ATCOL forms a complex with substances through electrostatic interaction and the resultant complex allows increased cellular uptake, resistance to degradation and prolonged release effect [10]. Thus, it seems that the ATCOL complex is potentially applicable for the efficient delivery of oligopeptides.

The purpose of this study was to evaluate the effect of ATCOL-carried anti-ubiquitination oligopeptide, Cblin (the ATCOL-Cblin complex) in starved C2C12 myotubes. The Cblin-ATCOL complex inhibited degradation of IRS-1 and dephosphorylation of Akt and Foxo3a in starved myotubes. Moreover, myotubes incubated with the Cblin-ATCOL complex showed resistance to starvation-induced atrophy. The results suggest that the Cblin-ATCOL complex can prevent muscle atrophy by maintaining proper IGF-1/Akt/FOXO signaling.

2. Materials and methods

2.1. Cell culture

C2C12 myoblastic cells were purchased from Dainippon Pharmaceutical Co. (Osaka, Japan) and maintained and proliferated at 37 °C under 5% CO_2–95% air in Dulbecco's modified Eagle's Medium (DMEM), supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. At 100% confluence, the cells were fused by replacing the medium with DMEM containing 2% horse serum. The cells were maintained in 2% house serum (differentiation medium) before the experiments. In starvation studies, the medium of the differentiated myotubes was replaced with serum-free medium for 24 h of incubation (serum starvation).

2.2. Preparation of the Cblin-ATCOL complex

The anti-ubiquitination oligopeptide (Cblin: Cbl-b inhibitor) is a pentapeptide corresponding to amino acids 606 to 610 of rat IRS-1 (DGpYMP) [17], synthesized by Thermo Fisher Scientific (Dreieich, Germany). C2C12 myotubes were transfected with Cblin using ATCOL (AteloGene®, KOKEN, Tokyo, Japan), 3 h before starvation. Cblin was used at a final concentration of 75.6 μM with 5% ATCOL. The Cblin-ATCOL complex taken up into C2C12 myotubes in the differentiation medium was observed using Cblin with FITC to the N-terminal under BIOREVO BZ-9000 fluorescent microscope (Keyence, Osaka, Japan). The complex was prepared by mixing ATCOL at various amounts of 100, 200, 400 μL with a fixed amount of 100 μg of Cblin and the mixture was added to 2 ml differentiation medium. The uptake efficiency of Cblin-ATCOL complex into C2C12 myotubes was maximal when 100 μL ATCOL was mixed with 100 μg Cblin (Fig. S1). Honma et al. [10] indicated that ATCOL establishes a complex with various physical morphologies depending on its amount and that excess ATCOL formed a complex with fibrous morphology leading to deterioration of transfection efficiency into cultured cells. Therefore, we used the above proportion of Cblin and ATCOL in the present experiment. The unphosphorylated Cblin (DGYMP) was used as a control-peptide, which has no inhibitory effect on IRS-1 ubiquitination, as reported in our previous study [17].

2.3. Measurement of myotube diameter

Myotubes were photographed at ×20 magnification after 24 h of serum starvation using BIOREVO BZ-9000 fluorescent microscope (Keyence). At least 100 diameters were measured per group using BIOREVO BZ-9000 software (Keyence).

2.4. Immunoblotting analysis

Cells were prepared in 50 mM Tris–HCl buffer (pH 7.5) containing 150 mM NaCl, 1% Triton X-100, 5 mM EDTA, 10 μM epoxomicin, 10 mM NaF, 2 mM Na_3VO_4, and a cocktail of protease inhibitors (Roche Diagnostics, Tokyo, Japan). The samples were homogenized using a sonicator. The BCA Assay (Pierce, Rockford, IL) was used to quantify proteins. Protein samples were combined with 4× sample buffer (250 mM Tris–HCl, 8% SDS, 40% glycerol, 8% beta-mercaptoethanol, 0.02% bromophenol blue) and separated onto polyacrylamide gel. The proteins were transferred to a polyvinylidene difluoride membrane and probed with the primary antibody according to the instructions provided by the manufacturer; the antibodies used were rabbit polyclonal anti-IRS-1 (Millipore, Bedford, MA), mouse monoclonal anti-Cbl-b (Santa Cruz Biotechnology, Santa Cruz, CA), mouse monoclonal anti-Akt (BD Bioscience, San Jose, CA), rabbit polyclonal phosphor-Akt (Ser473) (Cell Signaling Technology, Danvers, MA), rabbit monoclonal anti-FOXO3a (Cell Signaling Technology), rabbit polyclonal anti-phospho- FOXO3a (Thr32) (Cell Signaling Technology), mouse monoclonal skeletal fast-type MHC (Sigma, St. Louis, MO), and mouse monoclonal anti-β-actin (Sigma). Secondary antibodies were anti-rabbit (dilution, 1:5000) and anti-mouse (dilution, 1:5000) (Amersham Pharmacia Biotech, Piscataway, NJ). Membranes were developed using Amersham™ ECL™ western blotting detection reagents (GE Healthcare, Buckinghamshire, UK).

2.5. Immunoprecipitation analysis

Cells were prepared using 500 μg protein and adjusted to 300 μL with 50 mM Tris–HCl buffer (pH 7.5) containing 150 mM NaCl, 1% Triton X-100, 5 mM EDTA, 10 μM epoxomicin, 10 mM NaF, 2 mM Na_3VO_4, and a cocktail of protease inhibitors. They were incubated with either 0.5 μL of primary antibody at 4 °C for 3 h with rotation. Thirty μL of Protein G Sepharose™ 4 fast flow (GE Healthcare) were added to each IP sample and rotated at 4 °C for 16 h. Samples were washed 6 times in wash buffer, after which samples were prepared for SDS–PAGE as described above.

2.6. Immunofluorescence analysis

Cells were fixed in 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 and then blocked for 1 h at room temperature. Next, the cells were incubated with primary antibody: rabbit polyclonal anti-FOXO3a/FKHRL1 (Millipore) and mouse monoclonal skeletal fast-type MHC (Sigma) overnight at 4 °C, followed by incubation with the secondary antibody: Alexa Fluor 568 goat anti-rabbit IgG and Alexa Fluor 488 goat anti-mouse IgG (Life Technologies, Gaithersburg, MD) for 1 h at room temperature. Finally, the cells were incubated with Hoechst 33342 for 10 minutes at room temperature and examined under BIOREVO BZ-9000 fluorescent microscope (Keyence).

2.7. Real-time RT-PCR

Total RNA was extracted from whole cells according to standard protocols [3]. Real-time RT-PCR with SYBRTM Green dye was performed using an ABI 7300 real-time PCR system (Applied Biosystems, Foster City, CA) as described previously [9]. The following oligonucleotide primers were used for amplification: 5′-GGCG