Effects of Serum Deprivation on Expression of Proteolytic-Related Genes in Chick Myotube Cultures

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We previously reported that serum deprivation stimulates myofibrillar proteolysis in chick myotubes. In the present study, we examined the effect of serum deprivation on expression of the proteolytic-related genes (ubiquitin, proteasome, calpains, and cathepsin B) by real-time PCR of cDNA in chick myotubes. Myotubes were incubated with serum-free medium for 24 h. Ubiquitin and proteasome subunits (C1 and C2) and calpains (m-, μ-, and p94/calpain-3) but not cathepsin B mRNA expression were increased by serum deprivation. These results indicate that serum deprivation stimulates ubiquitin-proteasome and calpain proteolytic pathways, resulting in an increase in myofibrillar proteolysis in chick myotubes.

Key words: myotubes; calpain; ubiquitin; proteasome; serum deprivation

It has been established that supplementation of animal serum into the culture medium is required to cultured mammalian and avian cells in vitro. One of the reasons why animal serum is required has been shown that a large number of growth factors, which are necessary to induce cell growth and development, exist in the serum. When cultured cells are deprived of serum, the rate of intracellular proteolysis increases. We previously reported that serum deprivation induces myofibrillar proteolysis in chick myotubes.

Cells in culture show a series of changes in intracellular protein degradation in response to serum deprivation that is similar to alterations in degradation in tissues of starved animals. During starvation, proteolysis in muscle increases and helps provide the organism with essential amino acids for gluconeogenesis. However, the biochemical pathways for degradation of muscle protein and this mode of activation under starvation are still obscure.

Multiple proteolytic systems play a major role in various situations of protein loss and muscle wasting. Intercellular proteolytic processes found in skeletal muscle include various proteases such as lysosomal acidic cathepsins and Ca^{2+}-dependent calpains. Protein can also be degraded by the ATP-dependent ubiquitin-proteasome system. Ubiquitin-proteasome has been found to constitute an essential pathway a accelerated proteolysis in various animal models of muscle wasting. However, the precise roles of these degradation systems in the breakdown of skeletal muscle proteins are yet to be determined. Thus, in the present study, the effects of serum deprivation on proteolytic-related genes (ubiquitin, proteasome, calpains, and cathepsin B) expression in chick myotubes were investigated.

The cells were isolated from thigh muscle of 13-d-old chick embryos. Briefly, the muscle tissue obtained from the embryo was digested with dispase (Godo Shusei). The cell suspension was transferred to a 35 mm uncoated culture dish to allow fibroblast attachment. Cells were counted and plated onto gelatin-coated 12-wells plates (Iwaki Scitech) at a density of 2 x 10^5 cells/well. The cells were cultured in M-199 medium containing 15% calf serum (Invitrogen) and 2.5% chicken embryo extract (basal medium). The cells were grown at 37°C in a 5% CO2-enriched atmosphere of humidified air. The media were replaced every other day for the 7 d incubation period, and 90% of the cells formed myotubes at the seventh day. Myotubes were incubated for 24 h in the absence or presence of serum in M-199 medium (not containing chicken embryo extract). Total RNA was extracted using ISOGEN Reagent (Nippon Gene) according to the manufacturer’s protocols. cDNA was synthesized from 1–1000 ng of total RNA using random hexamer (TaKaRa, Tokyo, Japan) and Murine Moloney leukemia virus reverse transcriptase (Gibco Life Technologies, Gaithersburg, MD). Real-time PCR primers were designed (software Primer3, http://www.broad.mit.edu/cgi-bin/primer/primer3-www.cgi) for ubiquitin, the 20S proteasome C1 subunit, the 20S proteasome C2 subunit, the m-calpain large subunit, the μ-calpain large subunit, the p94/calpain-3 large subunit, cathepsin B, and GAPDH (Table 1). Gene expression was measured by real-time PCR using a LightCycler (Roche Diagnostics, Mannheim, Germany) instrument with the QuantiTect SYBR Green.
PCR system (Qiagen, Tokyo, Japan) starting with 5 ng of reverse-transcribed total RNA. GAPDH expression was used as an internal control. For quantification of the levels of mRNA expression, PCR product roughly equivalent in size and equivalent primer lengths and GC contents of each primer (50–60%) were selected. PCR was performed under the following conditions: 95 °C × 15 min, 45 × (94 °C × 15 sec, 55 °C × 20 sec, 72 °C × 15 sec).

Data were analyzed by Student’s t-test. A P value <0.05 was considered to be statistically significant. Each result is the mean ± standard deviation of the values obtained from six replicates.

When cultured cells are deprived of serum, the rate of intracellular proteolysis increases.

We previously reported that serum deprivation induces myofibrillar proteolysis (as measured by N2-methylohistidine release into medium) in chick myotubes. Doi et al. have also found that serum deprivation induces myofibrillar proteolysis and that insulin and thyroid hormones suppress serum deprivation-induced myofibrillar proteolysis in cultured muscle cells. The main purpose of the present experiments was to determine the proteolytic mechanism(s) involved in a serum deprivation-dependent increase in myofibrillar proteolysis. We measured the mRNA levels of components of the ubiquitin-proteasome system (non-lysosomal ATP-dependent proteolysis). The results of mRNA expression of ubiquitin and 20S proteasome subunits (C1 and C2) are also shown in Fig. 1. Ubiquitin mRNA expression was significantly increased by serum deprivation. The 20S proteasome C1 and C2 subunit mRNA expression was also increased significantly by serum deprivation. We also reported that serum deprivation stimulates proteasome activity in chick myotubes. The ATP-ubiquitin-proteasome-dependent pathway is activated by fasting as well as by the induction of stress hormones such as glucocorticoids that oppose the anabolic effects of insulin. These results are consistent with ours.

We next measured the mRNA levels for calpains of the non-lysosomal Ca2+-dependent proteolytic system. The results of mRNA expression of m-calpain, μ-calpain, and p94/calpain-3 large subunits are shown in Fig. 1. Calpains (m-, μ-, and p94/calpain-3) mRNA expression were significantly increased by serum deprivation. We also reported that serum deprivation stimulates calpain activity in chick myotubes. Calpain activation might trigger a dissociation of myofibrillar elements. It has been reported that m- and μ-calpain mRNA expression in skeletal muscle were increased by long-term starvation (8 d) in rabbits. We also found that short-term starvation increased m- and μ-calpain mRNA expression in skeletal muscles of chicks (unpublished data). Cells in culture show a series of changes in intracellular protein degradation in response to serum deprivation that is similar to alterations in degradation in tissues of starved animals. In our model, serum deprivation was found to be similar to alterations in the degradation of skeletal muscle in starved animals. The effect of serum deprivation on calpain expression in skeletal muscle had not been reported previously. We also found that serum deprivation increases p94/calpain-3 mRNA expression in chick myotubes. The predominant expression of p94/calpain-3 in skeletal muscle, where mRNA levels are about 10 times higher than those of m- and μ-calpain, indicates the physiological importance of p94/calpain-3 in that tissue. The muscle-specific calpain, p94/calpain-3, has binding sites on connectin/titin, raising the possibility that connectin/titin can be subjected to calcium-dependent proteolysis, thereby disrupting the anchorage of myosin to the Z-disk. Hayashi et al. have reported that glucocorticoid (corticosterone) stimulates calpain activity, resulting in a decrease in the connectin/titin content in the skeletal muscle of rats. The present

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence 5′–3′</th>
<th>Amplicon (bp)</th>
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<tbody>
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<td>Ubiquitin</td>
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<td>Reverse primer GCC TTC ACG TTC TCA ATG GT</td>
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<tr>
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<td>Forward primer ACG GTG TAC GAG CTG AGG AA</td>
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</tr>
<tr>
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<td>Reverse primer CTC TGC TGC TCC ACG TAC TA</td>
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<tr>
<td>C2</td>
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<td>241</td>
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<tr>
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<tr>
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<tr>
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<tr>
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<td>GAPDH</td>
<td>Reverse primer GGG CTA CAA ACT GAC CCC ATT CAG AG</td>
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Table 1. Gene Sequences Used as Forward and Reverse Primers for Real-Time PCR

| Last column indicates the length of amplicon (bp). C1, 20S proteasome C1 subunit; C2, 20S proteasome C2 subunit; m-calpain, m-calpain large subunit; μ-calpain, μ-calpain large subunit; p94/calpain-3, p94/calpain-3 large subunit. |
study provides the first evidence that serum deprivation might be associated with increased expression of p94/calpain-3.

We next measured the mRNA level for cathepsin B of the lysosomal-dependent proteolytic system. The result of the mRNA expression of cathepsin B is also shown in Fig. 1. Cathepsin B mRNA expression was not increased by serum deprivation. Lysosomal cathepsin B and L are endopeptidases thought to play major roles in intracellular protein degradation. Lysosomal proteases can degrade sarcoplasmic proteins but not myofibrillar proteins. Ilian and Forsberg have also reported that the cathepsin D mRNA expression of skeletal muscle was increased by long-term starvation (8 d) in rabbits. It has been reported that cathepsin L and D mRNA expressions encoding cathepsin B and D stimulate myofibrillar degradation in rabbit skeletal muscles in vitro. However, Medina et al. reported that fasting did not stimulate cathepsin L and D mRNA expressions in rat skeletal muscles. Bertile et al. also reported that short-term fasted rats did not increase in levels of skeletal muscle mRNAs encoding cathepsin B and D. Cells in culture show a series of changes in intracellular protein degradation in response to serum deprivation similar to alterations in degradation in tissues of starved animals. In the present experiment, chick myotubes were incubated with serum-free medium for short durations (24 h). Cathepsin B mRNA expression might not be increased by serum deprivation in chick myotubes during short-term incubation. In this study, we did not measure cathepsin L or D mRNA expression. Serum deprivation might have no effect on cathepsin L or D mRNA expression in chick myotubes, though this was not determined in the present study. It is necessary to examine the effect of serum deprivation on cathepsin L and D mRNA expression in chick myotubes.

Wing et al. have reported that lysosomal-dependent proteolysis was increased in skeletal muscles by fasting. They found that lysosomal proteolysis increases in muscle proteolysis during fasting using incubated muscle inhibited with methylamine-containing medium. They did not measure cathepsin mRNA expression. We previously also reported that serum deprivation stimulates cathepsin B+L and D activities in chick myotubes. However, increased levels of mRNA for various enzymes do not necessarily reflect increased proteolytic activity, indicating that there is no necessary relationship between mRNA levels and protein degradation.

The involvement of the different proteolytic systems in muscle wasting has been extensively studied in various animal models. In fasting, the ATP-ubiquitin-proteasome-dependent pathway is activated as well as a consequence of the induction of the stress hormone like glucocorticoids that oppose the anabolic effects of insulin. Calpain-dependent proteolysis is also elevated during fasting in skeletal muscles. In this study, serum deprivation induced ubiquitin proteasome, and calpain mRNA expression in chick myotubes.
Like other tissues, skeletal muscle contains multiple proteolytic systems, each of which could play an important role in muscle atrophy. Ubiquitin-proteasome (non-lysosomal ATP-dependent proteolysis), calpains (non-lysosomal Ca\(^{2+}\)-dependent proteolysis), and cathepsins (the main agents of lysosomal degradation) have been well established as contributing to muscle protein breakdown. In the present experiment, we measured ubiquitin, proteasome, calpains, and cathepsin B mRNA expression, and found that serum deprivation induces calpain, ubiquitin-proteasome proteolytic pathways in chick myotubes. This finding confirms that serum deprivation induces multiple proteolytic pathways in chick myotubes.

In conclusion, the present study shows that serum deprivation stimulates ubiquitin-proteasome and calpain proteolytic pathways, resulting in an increase in myofibrillar proteolysis in chick myotubes.

References
