Contribution of the autophagy-lysosomal and ubiquitin-proteasomal proteolytic systems to total proteolysis in rainbow trout (Oncorhynchus mykiss) myotubes

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Seiliez I, Dias K, Cleveland BM. Contribution of the autophagy-lysosomal and ubiquitin-proteasomal proteolytic systems to total proteolysis in rainbow trout (Oncorhynchus mykiss) myotubes. Am J Physiol Regul Integr Comp Physiol 307: R1330–R1337, 2014. First published October 1, 2014; doi:10.1152/ajpregu.00370.2014.—The ubiquitin-proteasome system (UPS) is recognized as the major contributor to total proteolysis in mammalian skeletal muscle, responsible for 50% or more of total protein degradation in skeletal muscle, whereas the autophagic-lysosome system (ALS) plays a more minor role. While the relative contribution of these systems to muscle loss is well documented in mammals, little is known in fish species. The current study uses myotubes derived from rainbow trout myogenic precursor cells as an in vitro model of white muscle tissue. Cells were incubated in complete or serum-deprived media or media supplemented with insulin-like growth factor-1 (IGF-1) and exposed to selective proteolytic inhibitors to determine the relative contribution of the ALS and UPS to total protein degradation in myotubes in different culture conditions. Results indicate that the ALS is responsible for 30–34% and 50% of total protein degradation in myotubes in complete and serum-deprived media, respectively. The UPS appears to contribute much less to total protein degradation at almost 4% in cells in complete media to nearly 17% in serum-deprived cells. IGF-1 decreases activity of both systems, as it inhibited the upregulation of both proteolytic systems induced by serum deprivation. The combined inhibition of both the ALS and UPS reduced degradation by a maximum of 55% in serum-deprived cells, suggesting an important contribution of other proteolytic systems to total protein degradation. Collectively, these data identify the ALS as a potential target for strategies aimed at improving muscle protein retention and fillet yield through reductions in protein degradation.

protein turnover; proteolysis; proteasome; autophagy

WHITE MUSCLE represents ~50% of rainbow trout (Oncorhynchus mykiss) weight, and the dry biomass of this tissue is predominantly protein. Nutrient modeling suggests the rate of protein accretion in white muscle is the main determinant of the rate of live weight gain (10). Protein accumulation is dependent on both rates of protein synthesis and protein degradation, with protein accumulation requiring rates of protein synthesis to exceed that of degradation. Approximately 76% of the proteins synthesized in rainbow trout white muscle are retained as growth (28, 54). Therefore, regulation of protein degradation has the potential to significantly affect growth rates and protein retention, so characterizing proteolytic mechanisms is central to understanding and improving growth and nutrient retention.

Protein degradation is regulated by numerous factors, including developmental age, with a significant upregulation during energy-intensive processes like sexual maturation (14) and spawning migration (41), and changes in nutrient supply, with increased protein degradation associated with feed restriction (12) and feed deprivation (17, 49, 51). Additional in vitro and in vivo research has identified biological factors that directly affect rates of protein degradation in skeletal muscle. These studies identified the ubiquitin-proteasome system (UPS) and the autophagy lysosome system (ALS) as proteolytic mechanisms that exhibit the greatest levels of regulation (15, 48, 49). Therefore, changes in protein degradation in muscle are likely attributed to regulation of these systems rather than regulation of proteolysis via the calpains and inhibitory calpastatins.

Inhibition of proteasome activity in mammalian muscle tissue or muscle-derived cell culture using proteasome-specific inhibitors reduced rates of protein degradation by ~40–50% (6, 60), although reductions of up to 90% are reported in other cell types (45). These findings suggest that this pathway is responsible for the majority of protein degradation in muscle. Protein degradation via the proteasome requires initial polyubiquitination of the substrate proteins targeted for degradation through a series of reactions involving E1, E2, and E3 enzymes critical for ubiquitin activation, conjugation, and protein substrate labeling, respectively (7). Over six hundred E3 ubiquitin ligases have been annotated from the human genome, and they function to polyubiquitinate misfolded proteins or specific proteins or protein families (36). The capacity for E3 ubiquitin ligases to target specific proteins for ubiquitination suggests that this mechanism provides for selective degradation of specific proteins, allowing for the precise regulation of cellular functions (3). Therefore, regulation of UPS can occur at multiple levels, including both the capacity for ubiquitination to regulation of components of the proteasome machinery. In salmonid muscle most research has focused on regulation of ubiquitin ligase expression, with a focus on FBXO32, which plays a role in muscle growth. In mammals Pbxo32 interacts with proteins that function in muscle growth and development like MyoD (57), myogenin (29), and eukaryotic initiation factor 3 subunit-f (eIF3-f) (19). In salmonid muscle expression of Pbxo32 increases dramatically (14- to 600-fold) during catabolic states (8, 13, 14) and decreases during anabolic states (16), with insulin-like growth factor-I (IGF-1) and insulin as negative regulators of this gene (15).
In contrast to the UPS, the ALS represents a more nonselective route of proteolysis that is responsible for ~40% of protein degradation in mammalian muscle (60). During autophagy, portions of cytoplasm and cell organelles are sequestered into vesicles called autophagosomes. The fusion of autophagosomes with lysosomes is followed by digestion of vacuole contents by lysosomal hydrolases such as cathepsins. In rainbow trout, indices of ALS activity increase during catabolic states like feed deprivation (49) and sexual maturation (14, 40), while amino acids are negative regulators of this system (48). In mammals IGF-1-induced inhibition of autophagy is regulated in part by phosphorylation and inactivation of FOXO1 and FOXO3a, which reduces expression of FOXO1 and FOXO3 phosphorylation but has a low or no effect on autophagy-related gene expression, suggesting that alternative mechanisms are in place to mediate this effect (49, 52).

Unique aspects of protein degradation in fish suggest that the relative contribution of each proteolytic system to total protein degradation may differ compared with what is observed in mammals. Additionally, salmonids derive much of their energy from amino acids and lipids rather than carbohydrates, suggesting that endogenous amino acid recycling differs from mammalian species that often favor carbohydrates as energy sources. The current study uses primary cell cultures derived from rainbow trout myosatellite cells as an in vitro model of white muscle tissue. Cells are exposed to selective proteolytic inhibitors to determine the relative contribution of the ALS and UPS to total protein degradation in myotubes. In addition, myotubes are serum deprived or serum deprived with IGF-1 to characterize the contribution of the major proteolytic systems to total protein degradation in myotubes. Inhibition of ALS activity in myotubes IGF-1 induces FoxO3 phosphorylation but has a low or no effect on autophagy-related gene expression, suggesting that alternative mechanisms are in place to mediate this effect (49, 52).

Inhibitor treatments. Stock solutions (1000×) of each inhibitor were made using DMSO and stored at −20°C. Solutions were diluted to a final concentration of 1× in media, and final DMSO concentrations in media never exceeded 0.02%. Control wells received equal volumes of DMSO compared with treatment wells. Final concentrations (1×) of the proteasome inhibitor carfilzomib (CFZ) (Selleckchem, no. S2853) and the autophagy inhibitor bafilomycin A1 (Baf A1) (Santa Cruz Biotechnology, sc-201550) were both 100 nM. MG132, the inhibitor of multiple proteolytic systems including the proteasome, cathepsins, and calpains, was used at a final concentration of 10 μM (Selleckchem, S2619). The optimal concentration of CFZ was determined based on maximal inhibition of proteasome chymotrypsin activity in liver homogenates, which was detected fluorometrically by cleavage of AMC-linked peptides. Maximum inhibition averaged ~85% for 100 nM CFZ (data not shown), and this concentration and amount of inhibition is consistent with reported effects in mammalian cells (33). Baf A1 has been previously shown to be effective at inhibiting autophagy in rainbow trout primary cell culture at 100 nM (48).

Protein degradation assay. Protein degradation assays were performed in triplicate, with each replicate identified as an independent cell isolation event. Each treatment was performed in triplicate wells within each assay. Rates of protein degradation were determined by modifying procedures developed in rainbow trout and mammalian cell culture systems (15, 27). Five-day-old cells were incubated for 2 days in complete media containing 2.5 μCi [3,5-3H]tyrosine (BP Biochemicals) per milliliter. On day 7 radioactive media was removed and cells were washed twice with Hanks balanced salt solution (HBSS). Cells were then incubated with complete media + 2 mM tyrosine for 2 h. Media was removed and cells were washed twice with HBSS. Treatments were applied and after incubation (5 or 10 h, as indicated), 500 μl of media were removed and added to 500 μl cold 20% trichloroacetic acid (TCA) and incubated at 4°C for 1 h. The mixture was centrifuged at 13,000 g for 10 min, and radioactivity in the acid soluble supernatant was quantified using liquid scintillation counting. In mammalian cells after the removal of 500 μl media, the remaining media was removed and the cell layer was washed twice with cold HBSS. Cells were layered with 300 μl 10% TCA and incubated at 4°C for 1 h. Wells were scraped and precipitated protein was pelleted by centrifugation at 13,000 g for 10 min. The acid-insoluble protein pellet was resuspended in cell lysis solution (0.1% Triton-X in 0.1 N NaOH) and counted for radioactivity using liquid scintillation counting. Total radioactive protein was calculated as the sum of the radioactivity in the TCA soluble and insoluble fractions. Protein degradation was expressed as [3,5-3H]tyrosine released into the media (TCA soluble) as a percentage of total [3,5-3H]tyrosine incorporated into cells (total radioactive protein).

Immunoblot assay. Western blot assays were performed in duplicate, with each replicate identified as an independent cell isolation event. Each treatment was performed in triplicate wells within each assay. Cells were incubated with treatment media for 5 h. Wells were washed with cold PBS and layered with 60 μl lysis buffer (150 mM NaCl, 10 mM Tris-HCl, 1 mM EGTA, 1 mM EDTA, 100 mM NaF, 4 mM sodium pyrophosphate, 1% Triton-X, 0.5% IGEPA L CA-30, and 1X Halr protease inhibitor cocktail, pH 7.5–7.6) and incubated on ice for 15 min. Wells were scraped and cell lysate was harvested and stored at −20°C. Protein concentrations were determined with the Bradford reagent method (9). Cell lysates (10 μg of protein) were subjected to SDS-PAGE and Western blot analysis using the appropriate antibody: anti- phospho S6 (Ser235/Ser236) (Cell Signaling Technologies, 4856), anti-carboxyl terminal S6 (Cell Signaling Technologies, 2217), anti-LC3B (Cell Signaling Technologies, 2775), anti-polyubiquitin proteins (Merck Millipore, 04-262), and anti-

MATERIALS AND METHODS

Cell culture. Rainbow trout were raised according to standard operating procedures and harvested using procedures approved by the National Center for Cool and Cold Water Aquaculture (NCCCAW) Institutional Animal Care and Use Committee (protocol no. 45). Myogenic precursor cells were isolated using a described modification (15) of a previously published procedure (23). Briefly, muscle tissue was removed from young rainbow trout (5–20 g) and collected into ice-cold suspension media (DMEM, 9 mM NaHCO3, 20 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.50 μg/ml amphotericin). Muscle tissue was minced and treated with a 0.2% collagenase solution, followed by a 0.1% trypsin treatment. The cell suspension was filtered successively through cell strainers (100 μm, 70 μm, and 40 μm), and cells in the flow through were collected by centrifugation (150 g, 10 min, 4°C). The pellet containing myogenic precursor cells was resuspended in complete media (suspension media containing 2.5% FBS), and the cells were counted and diluted to a concentration of 1.5–1.75 × 10⁶ cells/ml. One milliliter of resuspended cells was plated into each well of 12-well plates that were pretreated with poly-L-lysine and laminin. After at least 1.5 h, wells were gently washed with phosphate-buffered saline (PBS), and the adhered cells were layered with fresh complete media. Cells were incubated at 18°C under ambient air and complete media was replaced every other day. Treatments were applied to 7-day-old cells, which have been previously characterized as myotubes (25), and this was confirmed by visual evaluation of cell morphology.

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**RESULTS**

To establish serum deprivation as a suitable model for regulation of protein degradation, cells were incubated in media with increasing amounts of FBS (0–10%) for 5 h. Rates of protein degradation increased with decreasing amounts of serum, with the 10% FBS treatment exhibiting ~20% lower rates of protein degradation compared with complete serum deprivation (Fig. 1A). Since the 0% and 10% treatments produced the greatest difference in protein degradation, effects of serum deprivation in additional experiments were evaluated against cells incubated in media containing 10% FBS. Figure 1B indicates the relative abundance of pS6 to S6, which is a marker for anabolic activity. As expected, serum deprivation decreased the ratio of pS6 to S6, again confirming a physiological response to this treatment.

We then tested the feasibility to specifically block both the UPS and the ALS systems in our cell culture model. For the former, we used CFZ, which irreversibly binds to and inhibits the chymotrypsin-like activity of the 20S proteasome. As expected, treatment with CFZ resulted in the accumulation of polyubiquitinated proteins in 10% FBS-, 0% FBS-, and IGF-1-incubated cells, indicating an inhibition of the UPS system (Fig. 2A). With CFZ treatment, serum-deprived cells exhibited greater abundance of polyubiquitinated proteins compared with the 10% FBS- and IGF-1-treated cells, indicating an upregulation of the UPS during serum deprivation was prevented with IGF-1 treatment. To inhibit the ALS system we treated cells with Baf A1, a vacuolar ATPase inhibitor that inhibits autophagosome-lysosome fusion and prevents the degradation of ALS substrates including the well-known autophagosomal marker LC3II. As expected, treatment with Baf A1 increased abundance of LC3II in all treatments, indicating an inhibition of the ALS-mediated protein degradation (Fig. 2B). In the presence of Baf A1 serum deprivation increased abundance of LC3II, reflecting upregulation of the ALS, which was prevented with the addition of IGF-1 (Fig. 2B). The specificity of each proteolytic inhibitor was also evaluated by incubating cells in serum-depleted media containing an inhibitor, followed by Western blot analysis for the proteolytic pathway not intended for inhibition. Figure 2C indicates abundance of polyubiquitinated proteins was not affected by treatment with Baf A1 for 5 h. Similarly, abundance of LC3II was not affected by treatment with CFZ for 5 h (Fig. 2D), indicating that inhibitors did not have unintended effects on nontarget proteolytic pathways.

Having validated the efficiency as well as the specificity of CFZ and Baf A1 in our cell culture model, we then monitored the effect of serum depletion or serum depletion with IGF-1 supplementation on protein degradation rate in cells incubated in the presence or absence of the inhibitors. Data presented in Fig. 3A are from cells incubated with media treatments containing inhibitors for the first 5 h of media treatments. Data presented in Fig. 3B are from cells incubated with media treatments for 10 h, with inhibitors added from 5 to 10 h. A sample of media was collected at 5 and 10 h, so data indicate rates of protein degradation only during the 5-h period cells were exposed to proteolytic inhibitors. When compared with 10% FBS, serum deprivation increased protein degradation by 1.25- to 1.3-fold during both time periods. Supplementing serum-deprived media with 100 nM IGF-1 reduced protein degradation to levels similar to the 10% FBS treatment (Fig. 3A), but this effect was transient as the degradation rate in IGF-1-treated cells was greater than the 10% FBS treatment after 5 h (Fig. 3B). Rates of protein degradation decreased to similar levels in all treatments when coincubated with Baf A1 for hours 0–5 (Fig. 3A). From the 5- to 10-h period, protein degradation rates in serum-deprived cells incubated with Baf A1 were greater than those incubated with 10% FBS plus Baf A1 but not IGF-1 plus Baf A1 (Fig. 3B). Treatment with CFZ decreased protein degradation in serum-deprived cells by 10–15% across both time periods, but this inhibitor did not affect protein degradation rates in 10% FBS-treated cells (Fig. 3, A and B). In IGF-1-treated cells, CFZ decreased protein degradation from 5 to 10 h but not 0 to 5 h after exposure to IGF-1 (Fig. 3, A and B).

The contributions of the ALS and UPS to total rates of protein degradation are shown in Table 1, as calculated from the difference between the control and inhibited treatments.
The ALS was upregulated ~1.3- to 1.45-fold during serum deprivation to account for 40–49% of total protein degradation, while its contribution to total protein degradation in cells in complete media was 30–34%. The UPS was upregulated approximately fivefold during serum deprivation, contributing 10–16% to total protein degradation, whereas in cells in complete media the contribution of the UPS was <4%. Supplementing serum-deprived media with IGF-1 decreased ALS activity to levels similar to cells in complete media. Similarly, IGF-1 decreased UPS to levels comparable to cells in complete media, but this effect was transient as the activity of the UPS increased after 5 h to account for almost 11% of total protein degradation.

The combined contribution of both the ALS and UPS to total rates of protein degradation was monitored by treating cells with both CFZ and Baf A₁ (Fig. 4). The results were compared with those obtained with the multiple proteolytic systems inhibitor MG132. The obtained results show that MG132 treatment reduced protein degradation by ~80% in cells incubated in 0% and 10% FBS, whereas the combination of CFZ and Baf A₁ reduced degradation by a maximum of 55% in serum-deprived cells.

**DISCUSSION**

The assessment of the activation rates of each proteolytic system requires the use of system inhibitors. In the case of the UPS, the abundance of polyubiquitinated proteins depends on the rate of their synthesis and disappearance, the latter occurring mainly through proteasomal degradation. Therefore, measuring the accumulation of polyubiquitinated proteins as an index of regulation of the UPS pathway is most accurate when their flux through the proteasome is blocked or at least significantly inhibited with CFZ. Likewise, LC3II itself is degraded during the autophagic process so immunoblot detection of LC3II in the presence of an inhibitor of autophagy, like Baf A₁, is established as a method to accurately detect regulation of the ALS (32, 39). According to this concept, immunoblots indicate serum deprivation upregulates both the ALS and UPS compared with cells in 10% FBS media. Supplementing serum-deprived media with 100 nM IGF-1 prevented the upregulation of both systems. Regulation of proteolytic systems indicated by immunoblot data are in agreement with results from protein degradation assays, collectively indicating upregulation of protein degradation with serum deprivation, which is preventable with IGF-1 supplementation. Use of proteolytic inhibitors in the protein degradation assay provides novel information regarding the contribution of each proteolytic system to total rates of degradation and the extent to which serum deprivation and IGF-1 regulate these systems.

Consistent across all treatments, the ALS is responsible for a significant portion of protein degradation, with this value averaging 30–34% in cells in complete media but reaching 50% during serum deprivation. However, the UPS appears to contribute much less to total protein degradation, at almost 4% in cells in complete media to nearly 17% in serum-deprived cells. One result of serum deprivation is the removal of growth factors that promote anabolic effects in cells; therefore, this treatment can represent conditions that promote catabolism and muscle wasting in vivo. However, the caveat to this concept is the assumption that myotubes behave and respond in vitro...
similar to muscle tissue in vivo, which may not always be the case. Results from this study regarding the serum deprivation response suggest that catabolic treatments such as feed deprivation, which is characterized by reduced plasma IGF-1 (24, 59), upregulate proteolytic activity of the ALS and UPS to increase muscle wasting. We speculate that plasma IGF-1 is a regulator of this effect as addition of IGF1 to serum-deprived cells reduced ALS and UPS activity to levels comparable to that of complete media.

Results in vitro suggest that the ALS is responsible for approximately two to three times more protein degradation than the UPS, suggesting that the ALS plays a greater role in protein degradation in rainbow trout myotubes and is the predominant route of protein degradation during serum deprivation. Although, values for the proteasome are likely underestimates as CFZ only achieves 85% inhibition of proteasome activity. Adjustment of protein degradation values to account for incomplete proteasome inhibition would increase the contribution of the proteasome by only a minor amount (1.15-fold). Although the UPS appears to contribute less to protein degradation, it was subject to greater regulation (5- to 7-fold) by serum deprivation and IGF1 compared with the ALS, which exhibited at most 1.5-fold change. The nature of the UPS being a substrate-specific degradation system suggests that its multifold regulation (both up and down) has a critical role in the treatment-specific physiological response on a mechanistic level. In contrast, if the ALS is responsible for 30% or more of total protein degradation, given the indiscriminate nature of this system, even a moderate amount of regulation can have substantial impacts on protein turnover in a cell or tissue.

In mammals estimates indicate the proteasome is responsible for a greater percentage of protein degradation than what was observed in salmonid muscle. Inhibition of proteasome activity in C2C12 myocytes using bortezomib reduced total protein degradation by 50%, with combined inhibition of the proteasome and lysosome reducing protein degradation by 90% (60). Like CFZ, bortezomib is a specific inhibitor of the proteasome, although it is less potent than CFZ as it inhibits proteasome chymotrypsin activity by 80% at 100 nM (31). Inhibitory activity of bortezomib was determined in rainbow trout white muscle homogenates and consistent with the mammalian system it was less potent than CFZ, reducing 20S proteasome activity by 75% (data not shown). A third proteasome inhibitor is MG132, and this compound can reduce protein degradation in C2C12 cells by 75% (47), although this is likely an overestimation of the contribution of the proteasome to total protein degradation since MG132 can also inhibit certain lysosomal cysteine proteases and the calpains (35). In the current study, CFZ was used because potency, but more important, selectivity of the inhibitor for the target proteolytic mechanism was critical.

Table 1. Percent contribution of the ALS and UPS to overall rates of protein degradation

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<th>Contribution to Total Protein Degradation, %</th>
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<td>0–5 h Treatment</td>
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<td>10% FBS</td>
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Data are means ± SE. ALS, autophagic-lysosome system; UPS, ubiquitin-proteasome system; FBS, fetal bovine serum; IGF1, insulin-like growth factor-1. Letters indicate a significant difference between means within the same inhibitor treatment and time period, $P < 0.05$. *Significant contribution to protein degradation (differs from 0%), $P < 0.05$. 

Fig. 4. Effect of inhibition of multiple proteolytic systems on protein degradation rates in rainbow trout myotubes. Cells were harvested after 5 h of incubation in treatment media in the presence or absence of inhibitors. Different letters indicate significant difference, $P < 0.05$. *Significant difference from uninhibited sample (0%), $P < 0.05$. 

Fig. 3. Effect of CFZ and Baf A1 on protein degradation in 0% FBS-, 10% FBS-, and IGF1-incubated cells 0–5 h (A) and 5–10 h (B) posttreatment exposure. Different letters indicate significant difference compared with other treatments, $P < 0.05$. 

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In mammals estimates indicate the proteasome is responsible for a greater percentage of protein degradation than what was observed in salmonid muscle. Inhibition of proteasome activity in C2C12 myocytes using bortezomib reduced total protein degradation by 50%, with combined inhibition of the proteasome and lysosome reducing protein degradation by 90% (60). Like CFZ, bortezomib is a specific inhibitor of the proteasome, although it is less potent than CFZ as it inhibits proteasome chymotrypsin activity by 80% at 100 nM (31). Inhibitory activity of bortezomib was determined in rainbow trout white muscle homogenates and consistent with the mammalian system it was less potent than CFZ, reducing 20S proteasome activity by 75% (data not shown). A third proteasome inhibitor is MG132, and this compound can reduce protein degradation in C2C12 cells by 75% (47), although this is likely an overestimation of the contribution of the proteasome to total protein degradation since MG132 can also inhibit certain lysosomal cysteine proteases and the calpains (35). In the current study, CFZ was used because potency, but more important, selectivity of the inhibitor for the target proteolytic mechanism was critical.
Combining proteasome and lysosome inhibitors in mammalian muscle cell cultures reduced protein degradation by 90%, whereas the combination of CFZ and Baf A1 in rainbow trout myotubes reduced degradation by a maximum of 55% in serum-deprived cells. An explanation for the incomplete inhibition of proteolysis is that the inhibitory capacity of Baf A1 and/or CFZ is underestimated, thereby underestimating the contributions of the target systems to total protein degradation. This is a possibility in cells treated with the CFZ as proteasome inhibitors are not capable of significant inhibition of all active sites within the proteasome, which include the 1) chymotrypsin-like, 2) trypsin-like, and 3) caspase-like sites. Coordinated inactivation of the chymotrypsin-like site and either the trypsin- or caspase-like sites is recommended as the most efficient approach to reducing proteasome activity in vivo (30). In HT-29 cancer cells, at 100 nM CFZ decreased chymotrypsin-like activity by greater than 80% and decreased caspase- and trypsin-like activities by ~60%, although only reductions in chymotrypsin-like activity were observed in vivo (21). While other inhibitors of chymotrypsin-like activity (e.g., bortezomib, salinosporamide A, and MG132) can also reduce activity of caspase- or trypsin-like sites, these compounds have off-target effects, like inhibiting nonproteosomal systems like calpains and lysosomal cathepsins (1, 11, 21, 46, 55, 58). While these compounds are effective at reducing protein degradation, they are less useful for the current study for determining the involvement of specific proteolytic systems.

An alternative explanation for remaining proteolysis is that additional proteolytic systems are responsible for almost half of protein degradation in trout muscle cells. In addition to the ALS and UPS, the calpain system is a third active route of proteolysis in muscle, although it lacks the capacity to degrade proteins into amino acids. Calpains can cleave numerous cytoskeletal proteins critical for muscle structure and function, including myofibrillar proteins like titin, tropomyosin, and troponin, but also several intracellular kinases and phosphatases involved in cell signaling (26). It is believed that an important function of the calpains is to disrupt the Z-disk in striated muscle, liberating actin and myosin and truncating the otherwise bulky cytoskeletal proteins for subsequent degradation via the UPS or ALS (18). Therefore, in the current study it is not possible to definitively attribute the remaining proteolysis to the calpain system, because it does not have the capacity to digest proteins into individual amino acids.

There is the possibility that CFZ is not inhibiting a significant percentage of proteasome activity, despite increasing polyubiquitinated protein abundance by two- to threefold. However, when serum-deprived cells were incubated with MG132, which inhibits calpains, lysosomal proteases, and chymotrypsin-, and trypsin-like proteasome active sites, protein degradation is reduced ~80%, whereas the additive degradation for CFZ and Baf A1 is ~55%. The difference between these two values may be partially attributed to reduced generation of peptide substrates for ALS and UPS degradation due to MG132 inhibition of calpain activity.

The contribution of the proteasome to total protein degradation appears lower in fish than what has been observed in mammalian systems. When compared with the rat, the high dependence of salmonids on crude protein and lipid as energy sources (42, 43) may contribute to differences in kinetics of protein turnover in muscle tissue. Rates of protein degradation in salmonid muscle are ~5–35% greater than what has been calculated in rats (28, 38), therefore, it is expected that proteolytic pathways are operating at a higher capacity for turnover of endogenous proteins. The lysosome is a nonenergy-dependent process, whereas the proteasome requires ATP; therefore, it may be energetically efficient for protein turnover to occur more through the ALS than the UPS. This concept could potentially describe the physiological rationale for the observed differences between rainbow trout and mammals in terms of relative contribution of each proteolytic pathway to total rates of protein degradation.

Reductions in protein degradation, especially in muscle tissue, can have positive effects on protein retention, muscle growth, and fillet yield. This has been shown in poultry, as a fast-growing line of chickens exhibit lower levels of muscle protein degradation at a young age compared with a control line (56). In salmonids, plasma IGF1 concentrations are often positively correlated with growth rate (22, 34, 44), and the current study suggests that the ability of IGF1 to reduce ALS-associated proteolysis, and to a lesser extent UPS-associated proteolysis, in muscle may be a physiological mechanism partially responsible for this correlation. In rainbow trout, due to the significant contribution of the ALS to total protein degradation, strategies that reduce the proteolytic activity of this system should have the greatest opportunity of increasing protein retention in muscle. Studies in rainbow trout and mammals indicate that amino acids reduce autophagy, partially through TOR (target of rapamycin)-dependent and -independent signaling pathways (37, 48). The extent of TOR activation depends on the type of amino acid, and this may potentially explain why some amino acids, like leucine, are more effective at regulating autophagy than others (2). In contrast, the ability of nonregulatory amino acids, like arginine, to reduce autophagy may represent TOR-independent mechanisms (2). The capacity for specific amino acids to regulate autophagy in rainbow trout muscle is largely unknown, although recent research demonstrates increased autophagy in methionine-deficient diets (5). These findings, in combination with evidence indicating amino acids can regulate ubiquitin ligase expression (5, 15) suggest that dietary amino acids can affect growth by directly regulating proteolytic pathways.

**Perspectives and Significance**

The major and minor contribution of the ALS and UPS, respectively, to total protein degradation in rainbow trout skeletal muscle differs from what has been observed in mammalian muscle. These findings indicate that the physiological relevance of the ALS and UPS systems also differ between these organisms, which may be partially explained by variation in protein degradation rates, dietary protein requirements, and susceptibility to muscle wasting. The capacity for amino acids to regulate autophagy suggests there is potential to directly reduce ALS-associated protein degradation through manipulation of the dietary amino acid profile. Given the potentially major role the ALS has in total protein turnover, reduced ALS activity through dietary means may be a strategy that improves protein retention, growth performance, and fillet yield. Central to this concept is future research that identifies the ability of specific amino acids or amino acid profiles to regulate the ALS.
Additional experiments are also warranted to define the relative contribution of each proteolytic system in vivo.

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