Effects of insulin-like growth factor-I, insulin, and leucine on protein turnover and ubiquitin ligase expression in rainbow trout primary myocytes

Beth M. Cleveland and Gregory M. Weber
United States Department of Agriculture, Agricultural Research Service, National Center for Cool and Cold Water Aquaculture, Kearneysville, West Virginia

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Cleveland BM, Weber GM. Effects of insulin-like growth factor-I, insulin, and leucine on protein turnover and ubiquitin ligase expression in rainbow trout primary myocytes. Am J Physiol Regul Integr Comp Physiol 298: R341–R350, 2010. First published December 9, 2009; doi:10.1152/ajpregu.00516.2009.—The effects of insulin-like growth factor-I (IGF-I), insulin, and leucine on protein turnover and pathways that regulate proteolytic gene expression and protein polyubiquitination were investigated in primary cultures of 4-day-old rainbow trout myocytes. Supplementing media with 100 nM IGF-I increased protein synthesis by 13% (P < 0.05) and decreased protein degradation by 14% (P < 0.05). Treatment with 1 μM insulin increased protein synthesis by 13% (P < 0.05) and decreased protein degradation by 17% (P < 0.05). Supplementation media containing 0.6 mM leucine with an additional 2.5 mM leucine did not increase protein synthesis rates but reduced rates of protein degradation by 8% (P < 0.05). IGF-I (1 nM–100 nM) and insulin (1 nM–1 μM) independently reduced the abundance of ubiquitin ligase mRNA in a dose-dependent manner, with maximal reductions of ~70% for muscle atrophy F-box (Fbx) 32, 40% for Fbx25, and 25% for muscle RING finger-1 (MuRF1, P < 0.05). IGF-I and insulin stimulated phosphorylation of FOXO1 and FOXO4 (P < 0.05), which was inhibited by the phosphatidylinositol 3-kinase (PI 3-kinase) inhibitor wortmannin, and decreased the abundance of polyubiquitinated proteins by 10–20% (P < 0.05). Supplementing media with leucine reduced Fbx32 expression by 25% (P < 0.05) but did not affect Fbx25 or MuRF1 transcript abundance. Serum deprivation decreased rates of protein synthesis by 60% (P < 0.05), increased protein degradation by 40% (P < 0.05), and increased expression of all ubiquitin ligases. These data suggest that, similar to mammals, the inhibitory effects of IGF-I and insulin on proteolysis occur via PI 3-kinase/protein kinase B signaling and are partially responsible for the ability of these compounds to promote protein accretion.

PROTEIN ACCRUAL IS DEPENDENT ON RATES OF PROTEIN SYNTHESIS EXCEEDING RATES OF PROTEIN DEGRADATION. THESE RATES ARE DYNAMIC AND LARGELY INFLUENCED BY THE NUTRITIONAL AND PHYSIOLOGICAL STATE OF THE ANIMAL. EVIDENCE SUGGESTS THAT ANIMALS EXHIBITING BETTER FEED EFFICIENCY AND GROWTH PERFORMANCE ALSO DISPLAY SLOWER RATES OF PROTEIN DEGRADATION (15, 41, 82, 83). ADDITIONALLY, PROTEIN RETENTION EFFICIENCIES ARE HIGHEST AND FRAC TIONAL RATES OF PROTEIN DEGRADATION ARE SLOWEST IN YOUNG, FASTER-GROWING FISH (60). THEREFORE, DEVELOPING FEEDING STRATEGIES OR HUSBANDRY PRACTICES THAT OPTIMIZE GROWTH AND FEED EFFICIENCY NECESSITATE A BETTER UNDERSTANDING OF THE REGULATION OF PROTEOLYSIS IN THOSE ANIMALS.

WHEN RATES OF MUSCLE PROTEIN DEGRADATION SURPASS THOSE OF PROTEIN SYNTHESIS THEN MUSCLE ATROPHY OCCURS. EVIDENCE SUGGESTS THAT THE UBQUITIN-PROTEASOME PATHWAY IS RESPONSIBLE FOR THE MAJORITY OF PROTEIN DEGRADATION DURING MUSCLE ATROPHY (32, 36). AN INCREASE IN THE CAPACITY FOR PROTEIN DEGRADATION VIA THE PROTEASOME IS DEPENDENT ON AN INCREASE IN UBQUITIN LIGASE EXPRESSION (2, 72). UBQUITIN LIGASES ARE A FAMILY OF ENZYMES THAT ARE REQUIRED FOR THE TRANSFER OF AN ACTIVATED UBQUITIN MOLECULE TO A TARGETED PROTEIN, SUBSEQUENTLY MARKING THE PROTEIN FOR PROTEOSOMAL DEGRADATION (9). UBQUITIN LIGASES INCLUDE F-BOX TYPE PROTEINS, LIKE MUSCLE RING FINGER-1 (MURF1) AND MUSCLE ATROPHY F-BOX (FBX) PROTEINS SUCH AS THE MUSCLE SPECIFIC ATROGIN-1, OR FBX32 (2). UPREGULATION OF THESE ENZYMES HAS BEEN OBSERVED IN MAMMALIAN MODELS OF MUSCLE ATROPHY, SUCH AS THOSE CAUSED BY DENERVATION AND DISUSE (31, 67, 76), SEPSIS (18), AND GLUCOCORTICOID TREATMENT (81). ADDITIONALLY, FEED DEPRIVATION HAS BEEN DEMONSTRATED TO INCREASE EXPRESSION OF UBQUITIN LIGASES IN MUSCLE IN RAINBOW TROUT (ONCORHYNCHUS MYKISS) (7, 66, 74) AND OTHER ANIMALS (13, 29, 36, 53). IN RATS, THE UBQUITIN LIGASE FBX25 IS NOT UPREGULATED DURING MUSCLE ATROPHY (40). HOWEVER, IN TROUT, THE ABUNDANCE OF A TRANSCRIPT SIMILAR TO SALMON (ACCESSION NO.: NM_001139897.1) AND ZEBRAFISH (ACCESSION NO.: NM_205724.1) FBX25 INCREASES DURING FEED DEPRIVATION (74), SUGGESTING THAT THE REGULATION OF FBX HOMOLOGS DURING ATROPHY IS SPECIES-SPECIFIC.

SUPPRESSION OF UBQUITIN LIGASE EXPRESSION IS ESTABLISHED AS ONE OF THE MECHANISMS RESPONSIBLE FOR THE HYPERTROPHIC EFFECTS OF INSULIN-LIKE GROWTH FACTOR-I (IGF-I) AND INSULIN IN ALL VERTEBRATES INVESTIGATED (35, 68, 80), ALTHOUGH IT HAS NOT BEEN STUDIED IN FISH. IN MAMMALS, THESE HORMONES REDUCE PROTEIN DEGRADATION BY INITIATING SIGNALING THROUGH PHOSPHATIDYLINOSITIDE 3-KINASE (PI 3-KINASE) AND PROTEIN KINASE B (AKT) (35, 68, 86), WHICH LEADS TO THE PHOSPHORYLATION AND INACTIVATION OF FBOX OR FOXO TRANSCRIPTION FACTORS AND THEIR SUBSEQUENT TRANSLATION FROM THE NUCLEUS (28), THEREBY REDUCING TRANSCRIPTION OF FBX AND MURF1. THEREFORE, WHEN CIRCULATING IGF-I OR INSULIN CONCENTRATIONS ARE LOW, SIGNALING THROUGH PI 3-KINASE AND AKT IS DECREASED, REDUCING PHOSPHORYLATION OF FOXO, WHICH RESULTS IN UPREGULATION OF UBQUITIN LIGASE EXPRESSION AND PROTEIN POLYUBQUITINATION. IN ADDITION TO HORMONES, CERTAIN AMINO ACIDS ARE ALSO CAPABLE OF DECREASING EXPRESSION OF FBX AND MURF1 IN MAMMALS AND AVES (24, 52, 80), AND, AT LEAST FOR FBX, THIS OCCURS BY SIGNALING THROUGH THE TARGET OF RAPAMYCIN (TOR) PATHWAY, WHICH ALSO LEADS TO FOXO PHOSPHORYLATION (24). THEREFORE, THE HYPERTROPHIC EFFECTS OF IGF-I, INSULIN, AND AMINO ACIDS RESULT FROM THEIR ABILITIES TO INCREASE RATES OF PROTEIN SYNTHESIS AND DECREASE RATES OF PROTEIN DEGRADATION VIA DOWNREGULATION OF UBQUITIN LIGASES.

ALTHOUGH THIS CONCEPT IS WELL ESTABLISHED IN MAMMALS, THE EFFECTS OF IGF-I, INSULIN, AND AMINO ACIDS ON PROTEIN DEGRADATION HAVE NOT BEEN DEMONSTRATED IN FISH. IN FISH, STIMULATORY EFFECTS OF IGF-I ON PROTEIN SYNTHESIS HAVE BEEN REPORTED (21,
REGULATION OF PROTEIN DEGRADATION IN TROUT MYOCYTES

54), but it is unknown to what extent IGF-I regulates proteolysis. However, it has been shown in fish that insulin and IGF-I are capable of initiating signaling transduction through the PI 3-kinase/Akt pathway (4, 47, 63), and evidence suggests that IGF-I can regulate ubiquitin ligase and cathepsin expression (7). Additionally, amino acids were able to activate TOR signaling in rainbow trout (73), which is involved in increasing protein synthesis. Therefore, the hormones and amino acids that reduce protein degradation in mammals are capable of signaling through the critical pathways in fish, including rainbow trout.

It has been established in mammals that the anabolic effect of these compounds are in part defined by their ability to reduce protein degradation, but neither this reduction nor the mechanisms responsible for the reduction have been demonstrated in fish. In the present study, the effects of IGF-I, insulin, leucine, and serum deprivation on rates of protein turnover in rainbow trout were characterized. Additionally, the mechanisms responsible for these effects were investigated. These results will improve our understanding of the mechanisms responsible for the hypertrophic effects of anabolic hormones and amino acids in rainbow trout.

METHODS AND MATERIALS

Myosatellite cell isolation. All experimental procedures adhered to Animal Care and Use Committee (ACUC) guidelines and received approval from the National Center for Cool and Cold Water Aquaculture ACUC. Myosatellite cells were isolated by modifying a previously described procedure (17). Muscle tissue was removed from young rainbow trout (5–10 g) and collected in ice-cold suspension media (DMEM, 9 mM NaHCO3, 20 mM HEPES, 100 U/ml penicillin, and 100 μg/ml streptomycin). Muscle tissue was minced and resuspended in suspension media (1 g tissue/5 ml media) and centrifuged (300 g, 5 min, 4°C). The supernatant was discarded, and the pellet was resuspended in 0.2% collagenase (C9891, 1 g tissue/5 ml collagenase solution; Sigma-Aldrich, St. Louis, MO) in suspension media and gently agitated at 20°C for 1 h. The suspension was centrifuged (300 g, 20 min, 4°C), and the supernatant was discarded. The tissue pellet was resuspended in 0.1% trypsin (153571; MP Biomedicals, Irvine, CA) in suspension media and gently agitated for 45 min at 20°C. The suspension was then diluted 1:5 with suspension media and centrifuged (300 g, 20 min, 4°C). The supernatant was discarded, and the pellet was resuspended in suspension media and submitted to mechanical dissociation. The cell suspension was filtered successively through cell strippers (100 μm, 70 μm, and 40 μm), and cells in the flow through were collected by centrifugation (150 g, 10 min, 4°C). The resultant pellet containing the myosatellite cells was resuspended in complete media (suspension media with 10% FBS), and the cells were counted and diluted to a concentration of 10⁶ cells/ml. Cells were plated on a six-well plate to a concentration of 1.5–2.0 × 10⁶ cells/well. After 3 h, wells were gently washed with Hank’s buffered salt saline (HBSS), and the adhered myocytes cells were layered with fresh complete media.

Culture conditions. Culture dishes were pretreated with 100 μg/ml poly-L-lysine (P4832; Sigma-Aldrich) for 3 h at 18°C. Wells were then washed with sterile nanopure water and layered with 5 μg/ml laminin (L2020; Sigma-Aldrich) in PBS overnight at 18°C. Plates were wrapped in parafilm and stored at −80°C until use. Before cells were seeded, the laminin solution was aspirated, and the wells were washed with PBS. Cells were incubated at 18°C under ambient air, and fresh media was supplied every other day.

Treatment conditions. Protein degradation assays were performed on 4- and 7-day-old cell cultures, which exhibit the morphology of small and large myotubes, respectively. Protein synthesis, gene expression, and protein abundance analyses were performed on 4-day-old cell cultures. For gene expression and protein abundance analyses, cells were washed two times with HBSS and layerd with low-FBS media (DMEM, 9 mM NaHCO3, 20 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.5% FBS). After 2.5 h, low-FBS media was removed and replaced with media containing IGF-I, insulin, or leucine. Concentrations of IGF-I and insulin supplementation were 100 nM and 1 μM, respectively, which are concentrations known to exert responses in trout myocytes (4–6, 14). However, an IGF-I and insulin dose response was also completed, which included concentrations of IGF-I and insulin found in trout plasma. Any inhibitors were incubated with cells for 30 min before application of subsequent treatments. Recombinant human IGF-I (National Hormone & Peptide Program, Harbor-University of California Los Angeles, Torrance, CA) was used, which exerts similar binding properties and effects as recombinant trout IGF-I in rainbow trout cell culture (6, 8). Salmon insulin was a generous gift from Dr. Erika Pliisetskaya (University of Washington, Seattle, WA).

Protein synthesis. Rates of protein synthesis were determined by modifying procedures developed in mammalian cell culture (23). Four-day-old cells were incubated in treatment media containing 2.5 μCi [3,5-3H]tyrosine (MP Biomedicals)/ml media for 1 h. Media was removed, and the cells were washed with ice-cold HBSS, layered with 500 μl 10% TCA, and incubated at 4°C for 1 h. The wells were scraped, and the precipitated protein was pelleted by centrifugation at 9,600 g for 5 min. The acid-insoluble protein pellet was resuspended in cell lysis solution (1% Triton-X 100 and 1 N NaOH) and counted for radioactivity using liquid scintillation counting. The incorporation of tritium in the acid-insoluble cell fraction was linear between 0.31 and 7.5 μCi/ml media (data not shown) and with incubation times between 15 min and 4 h with [3,5-3H]tyrosine (data not shown).

Protein degradation. Rates of protein degradation were determined on 4- and 7-day-old cell cultures by modifying procedures developed in mammalian cell culture systems (23, 68). Two- or five-day-old cells were incubated for 2 days in complete media containing 2.5 μCi [3,5-3H]tyrosine/ml. Radioactive medium was removed, and cells were washed two times with HBSS + 2 mM tyrosine. Cells were then incubated with low-FBS media + 2 mM tyrosine for 2.5 h. Low-FBS media was removed, and cells were washed two times with HBSS. Treatments were applied, and, after 12 h of incubation, 500 μl of media were removed and added to 500 μl cold 20% TCA and incubated at 4°C for 1 h. The mixture was centrifuged at 9,500 g for 5 min, and the radioactivity in the acid-soluble supernatant was quantified using liquid scintillation counting. Immediately after removing 500 μl media, the remaining 1.5 ml media was removed, and the cell layer was washed two times with HBSS. Cells were layered with 500 μl 10% TCA and incubated at 4°C for 1 h. The wells were scraped, and the precipitated protein was pelleted by centrifugation at 9,600 g for 5 min. The acid-insoluble protein pellet was resuspended in cell lysis solution (1% Triton-X 100 and 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 in the media (TCA soluble) as a percentage of total [3,5-3H]tyrosine incorporated in cells (total radioactive protein). Protein degradation was measured to be linear over 24 h (data not shown), and equivalent degradation rates were observed whether 2.5 or 5.0 μCi [3,5-3H]tyrosine/ml media were used (data not shown).

Real-time RT-PCR. Treatment medium was removed, and cells were washed two times with HBSS. Trizol (1 ml; Invitrogen, Carlsbad, CA) was layered on cells, and total RNA was isolated per the manufacturer’s protocol. Quality and quantity of RNA was estimated using the ratio of absorbance at 260 to 280 nm. Next, 2 μg of RNA were DNase treated (Promega, Madison, WI) and reverse transcribed using random primers (Invitrogen) and Moloney murine leukemia virus reverse transcriptase (Promega) per the manufacturer’s protocol. The cDNA was diluted 1:4, and 2 μl were used in a 15-μl PCR...
Means are sons. All experiments were run in triplicate within duplicate wells. The least-significant-difference procedure was used for means comparison between treatment means. In the event of a significant difference, the PC-SAS general linear models procedure for significant differences was performed using Alpha Innotek software.

Antibodies were incubated in blocking buffer for at least 1 h. Membranes were then incubated with the membranes in blocking buffer overnight at 4°C. After incubation, membranes were washed two times with HBSS. Cells were then layered with 100 nM IGF-I, insulin, or leucine. In 4-day-old cells, IGF-I and insulin reduced rates of protein degradation by ~15% each, as measured by H3 release in culture media (Fig. 1). Supplementing media with 2.5 mM leucine reduced protein degradation by 7% relative to the control media. In 7-day-old cell cultures, IGF-I and insulin reduced rates of protein degradation by 28 and 19%, respectively (Fig. 1). The effects of leucine on protein degradation were not significantly different from control. Basal rates of protein degradation (percent of H3 released in culture media) in untreated control samples were greater in 4-day-old myocyte cultures (16.6 ± 0.52%) compared with 7-day-old myocyte cultures (14.1 ± 0.45%).

**Gene expression.** Primers were designed to two cDNA sequences (Table 1) obtained from the rainbow trout EST database that are similar to F-box proteins from other fish species. The primers identified as Fbx32 were designed from an EST with a region similar to the Fbx32 (MAFbx, atrogin-1) cDNA sequence in zebrafish. The primers identified as Fbx25 were designed from the consensus sequence of a contig that exhibits a high level of similarity to Fbx25 in both salmon and zebrafish. Primers designed to Fbx32, Fbx25, and MuRF1 transcripts generate PCR products from liver cDNA (unpublished data); therefore, none of these ubiquitin ligase transcripts are muscle-specific.

Myocytes were incubated in 100 nM IGF-I for 4 h, and the expression of ubiquitin ligases were monitored after 1, 2, and 3 days. Western blotting. Treatment medium was removed, and cells were washed twice with HBSS. Cells were then layered with 100 µl SDS sample buffer (62.5 mM Tris-HCl, 2% SDS, 10% glycerol, 50 mM dithiothreitol, 0.01% bromphenol blue) with protease and phosphatase (Thermo-Scientific, Waltham, MA) inhibitors, the wells were incubated in a boiling water bath for 5 min, and 20 µl of the denatured lysate were loaded onto a 4% stacking, 7.5% resolving SDS-polyacrylamide gel. Following SDS-PAGE, proteins were transferred to a nitrocellulose membrane, and membranes were incubated with blocking buffer (5% nonfat dry milk, 0.1% Tween 20 in TBS) for at least 1 h. The primary phospho-FOXO1(Ser199)/FOXO4(Ser263) (2487; Cell Signaling Technology), polyubiquinated protein (PW8805; BioMol International, Plymouth Meeting, PA), phospho-Akt(Thr308) (9275; Cell Signaling), and α-tubulin (T5168; Sigma) antibodies were incubated with the membranes in blocking buffer overnight at 4°C. After being washed, the membranes were incubated with appropriate secondary antibodies with blocking buffer for at least 1 h. Membranes were then washed, and proteins were visualized using chemiluminescent detection on radiography film. Bands were quantified by densitometry on a Fluorchem Alphalmager (Alpha Innotek, San Leandro, CA) with AlphaEase software (Alpha Innotek).

**Statistical analysis.** Data were analyzed by one-way ANOVA with a PC-SAS general linear models procedure for significant differences between treatment means. In the event of a significant F-value, the least-significant-difference procedure was used for means comparisons. All experiments were run in triplicate within duplicate wells. Means are ± SE.

**Table 1. Primers used for real-time RT-PCR**

<table>
<thead>
<tr>
<th>Gene Product</th>
<th>Primer Sequences (5’-3’)</th>
<th>Accession No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Actin*</td>
<td>GCCGGCCGCGCCTAGAGACTAC</td>
<td>AF157514</td>
</tr>
<tr>
<td>GAPDH</td>
<td>GCCGGCTGTTGCGTGAACCTGTAAC</td>
<td>AF027130</td>
</tr>
<tr>
<td>Fbx32*</td>
<td>TGGCATCAATGGATTTCAAAG</td>
<td>CX026010</td>
</tr>
<tr>
<td>Fbx25</td>
<td>CAAGCTTCAAGGGAGCTTCTC</td>
<td>TC141076</td>
</tr>
<tr>
<td>MuRF1</td>
<td>AGACTGCTACGGAGACCA</td>
<td>TC134460</td>
</tr>
<tr>
<td>Cathepsin D*</td>
<td>GCTGTGTTACATGATCAACT</td>
<td>U90321</td>
</tr>
<tr>
<td>Cathepsin L*</td>
<td>TGAAGGAGAAGATGTGGATGG</td>
<td>AF358668</td>
</tr>
</tbody>
</table>

**RESULTS**

**Protein synthesis.** Rates of protein synthesis were measured after 1 h of exposure to IGF-I, insulin, or leucine. Supplementing media with IGF-I or insulin increased the incorporation of tritium into the acid-insoluble cell fraction by 13% for each hormone (Fig. 1). However, supplementing media with 2.5 mM leucine, for a total media leucine concentration of 3.1 mM, did not result in faster rates of protein synthesis compared with control media.

**Protein degradation.** Rates of protein degradation were monitored in 4- and 7-day-old myocytes after 12 h of incubation with IGF-I, insulin, or leucine. In 4-day-old cells, IGF-I and insulin reduced rates of protein degradation by ~15% each, as measured by H3 release in culture media (Fig. 1). Supplementing media with 2.5 mM leucine reduced protein degradation by 7% relative to the control media. In 7-day-old cell cultures, IGF-I and insulin reduced rates of protein degradation by 28 and 19%, respectively (Fig. 1). The effects of leucine on protein degradation were not significantly different from control. Basal rates of protein degradation (percent of H3 released in culture media) in untreated control samples were greater in 4-day-old myocyte cultures (16.6 ± 0.52%) compared with 7-day-old myocyte cultures (14.1 ± 0.45%).
4 h (Fig. 2). The abundance of Fbx32 transcript was reduced significantly after just 1 h, and it continued to decrease to 25% compared with an untreated control after 4 h. Despite numerical reductions after treatment with 100 nM IGF-I for 1 h, the expression of Fbx25 and MuRF1 was significantly decreased only after 2 and 4 h of IGF-I treatment (Fig. 2). Incubation of myocytes in increasing concentrations of IGF-I for 4 h indicated that IGF-I regulates ubiquitin ligase expression in a dose-dependent manner (Fig. 3), with significant reductions starting at the lowest dose of 1 nM for Fbx32 and Fbx25 and 5 nM for MuRF1. Maximal reductions in transcript abundance occur at 5 nM IGF-I (38 ng/ml), which is within the range of IGF-I concentrations previously measured in plasma (34, 88), and persist up to 100 nM IGF-I (760 ng/ml).

Insulin was also able to reduce the abundance of all ubiquitin ligase transcripts in a dose-dependent manner (Fig. 4), with maximal reductions of Fbx32 occurring at 100 nM, Fbx25 at 1 μM, and MuRF1 at 5 nM. All transcripts exhibited a significant decrease at doses between 1 and 5 nM insulin, which fall within levels measured in plasma (73). Insulin at 1 μM decreased cathepsin expression to levels 0.83 and 0.89 of control for cathepsin D and cathepsin L, respectively (Table 2), whereas other treatments (100 nM IGF-I, 2.5 mM leucine, and serum deprivation) did not affect cathepsin expression.

In mammals, regulation of ubiquitin ligase expression is known to occur through the PI 3-kinase/Akt signal transduction pathway. Wortmannin, an inhibitor of PI 3-kinase that is effective in fish (4, 8), was used to inhibit signaling through this pathway, and the effect on ubiquitin ligase expression was determined. Exposing myocytes to wortmannin for 4 h resulted in a sixfold increase in Fbx32 transcript abundance (Fig. 5A), whereas increases in Fbx25 and MuRF1 were ~1.5-fold higher than a media control (Fig. 5B). Wortmannin prevented the IGF-I- or insulin-induced reduction in ubiquitin ligase expression, indicating that signaling through PI 3-kinase is required for the regulation of gene expression by IGF-I and insulin.

Treatment of myocytes with 2.5 mM leucine for 4 h reduced expression of Fbx32 by 27% but did not reduce expression of Fbx25 or MuRF1 (Fig. 6). Intracellular amino acid signaling is known to proceed through the TOR pathway, so rapamycin, an inhibitor of TOR that has been effective in fish (73), was used to determine how the absence of signaling through this pathway affects ubiquitin ligase expression. Exposing myocytes to rapamycin for 4 h increased expression of Fbx32 1.3-fold and prevented the leucine-induced reduction in Fbx32 expression (Fig. 6). Neither Fbx25 nor MuRF1 were affected by rapamycin treatment, suggesting that these genes are not regulated by amino acid signaling through TOR in rainbow trout myocytes. Additionally, cathepsin expression was not affected by leucine (Table 2).

Serum deprivation. To examine the effect of serum deprivation on ubiquitin ligase expression, myocytes were incubated in 10% FBS or serum-free media for 24 h, which was the time...
required for an effect on expression of all ubiquitin ligases. Serum deprivation increased Fbx32 and Fbx25 expression by ~1.3-fold, whereas MuRF1 expression increased 1.1-fold relative to cells incubated in 10% FBS (Fig. 7). Serum deprivation (12 h) resulted in a 1.4-fold increase in protein degradation (Fig. 7). The effect of serum deprivation was more pronounced on rates of protein synthesis, which decreased 60% compared with cells incubated in 10% FBS.

Protein abundance. In mammals, the effects of IGF-I and insulin on ubiquitin ligase expression are known to result from the phosphorylation of FOXO transcription factors. To determine if this mechanism exists in rainbow trout, the abundance of phosphorylated FOXO1 and FOXO4 was quantified in treated myocytes. Ubiquitin ligases function to ligate a ubiquitin molecule to a protein targeted for degradation; therefore, the abundance of polyubiquitinated proteins was also quantified. Treatment with IGF-I or insulin for 4 h doubled the abundance of phosphorylated FOXO1 and increased phosphorylation of FOXO4 3-fold and 1.5-fold, respectively (Fig. 8). The abundance of polyubiquitinated proteins significantly decreased 22 and 11% after 4 h of IGF-I and insulin treatment, respectively (Fig. 8). Treatment of cells with wortmannin for 30

Fig. 5. Effects of hormones and wortmannin (wort) on ubiquitin ligase mRNA expression. Cells were incubated in 0.5% FBS media containing the indicated treatments for 4 h before harvest. Cells that received treatments containing both wortmannin (1 μM) and hormones (100 nM IGF-I or 1 μM insulin) were treated with wortmannin 30 min before addition of hormones. Because of the short half-life of wortmannin in culture media (8–13 min), 2 μl of a wortmannin stock [1,000 X in dimethyl sulfoxide (DMSO)] were added to wells every 30 min throughout the 4-h incubation period. An additional control received 2 μl DMSO every 30 min to evaluate the effects of DMSO, which did not result in changes in ubiquitin ligase expression relative to the control 0.5% FBS media (data not shown). Changes in mRNA expression of ubiquitin ligases are shown relative to control media, which is set at 1.0. Data shown are means ± SE (n = 3). P < 0.05, significant differences from the control treatment (+).

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Fig. 6. Effects of leucine supplementation and rapamycin on ubiquitin ligase mRNA expression. Cells were incubated in 0.5% FBS media containing the indicated treatments for 4 h before harvest. Cells that received rapamycin (200 nM) and leucine (2.5 mM) were incubated with rapamycin for 30 min before leucine supplementation. Changes in mRNA expression of ubiquitin ligases are shown relative to control media, which is set at 1.0. Data shown are means ± SE (n = 3). P < 0.05, significant differences from the control media (*) and from the leucine treatment (×).

Fig. 7. Effect of serum deprivation (0% FBS) on protein turnover and ubiquitin ligase mRNA expression. Samples were incubated in 0.5% FBS media supplemented with 100 nM insulin-like growth factor-1 (IGF-I), 1 μM insulin, or 2.5 mM leucine for 4 h, or 0% FBS (serum deprivation) for 24 h. Changes in mRNA expression are shown relative to control media, which is set at 1.0. Control media contained either 0.5% FBS for IGF-I, insulin, and leucine treatments or 10% FBS for the serum deprivation treatment. *Significant differences from the control treatment, P < 0.05.
In fish, it is well-established that IGF-I stimulates growth (42, 90). This effect is attributed to the stimulatory effects of IGF-I and insulin on mitogenic and metabolic processes like oocyte maturation (87), cell proliferation (5, 46, 63), DNA synthesis (63), and nutrient uptake (5, 8, 14). The present study indicates that stimulation of protein synthesis and, for the first time in fish-derived cell culture, that reduction of protein degradation are also included as metabolic effects of IGF-I and insulin. These results are consistent with observations in rodent (10, 23, 37, 68), porcine (11), and avian (84) in vivo and myocyte cell culture studies that demonstrate analogous effects on protein turnover. In mammalian cardiomyocytes, which exhibit 4.5 times more insulin receptors than IGF-I receptors (57), IGF-I and insulin were equally effective at increasing rates of protein synthesis (19). However, in rainbow trout cardiomyocytes, which exhibit eight times more IGF-I receptors than insulin receptors (57), IGF-I increased protein synthesis, whereas insulin had the opposite effect (21), suggesting that, in fish, the effects of IGF-I and insulin on protein turnover are tissue specific.

In rainbow trout primary cell cultures, the concentration of IGF-I receptors increases as myocytes differentiate into myotubes (6). In the present experiment, the greater decrease in protein degradation rates during IGF-I treatment in the older, differentiated myotubes may be a result of increased IGF-I binding because of a higher receptor number, which may lead to a greater physiological response relative to smaller myotubes. Additionally, greater responses to IGF-I and insulin in differentiated myotubes are observed for glucose uptake, but not alanine uptake (5, 14). This may be reflective of changing metabolic conditions in differentiating cells as they transition from an anabolic to a maintenance state. The later state may favor amino acid recycling rather than protein accretion, which would require greater regulation of protein degradation and subsequently reduce the need for amino acid uptake.

The slower basal rates of protein degradation in 7-day-old cultures may be reflective of lower amino acid requirements because of slower growth and proliferation in the older cells. Because cells derive a portion of their free amino acids from endogenous protein degradation, a lower amino acid requirement may result in slower rates of protein breakdown. Additionally, the increased concentration of IGF-I receptors in older
cells may increase any effects of bovine IGF-I in the 0.5% FBS containing media and result in slower basal rates of protein degradation. However, the relative difference in rates of protein degradation between 4- and 7-day-old cultures may also be an effect of how cells of different ages respond to in vitro conditions. Therefore, it is difficult to determine exactly what contributes to the observed rate differences and extrapolate their significance to in vivo conditions where many metabolic factors that affect protein turnover are present.

At maximal doses, IGF-I and insulin were comparably effective at phosphorylating FOXO proteins, reducing ubiquitin ligase expression, increasing protein synthesis, and decreasing protein turnover. Unlike mammalian muscle, fish skeletal muscle has a higher abundance of IGF-I receptors than insulin receptors (1, 44, 57, 58). These IGF-I receptors also exhibit greater tyrosine kinase activity (4, 58) and greater specific binding (4, 57, 58) compared with insulin receptors. However, the ratio between these two measures indicates that insulin receptors possess greater phosphorylative capacity compared with IGF-I receptors (4), which may partially compensate for their lower receptor number. Nonetheless, at equimolar concentrations, IGF-I is more potent than insulin at stimulating GLUT1 expression (14), glucose (8) and amino acid uptake (5), and cell proliferation (5) in rainbow trout myocytes, suggesting that, in fish, IGF-I may have a more integrative role in metabolism and mitogenesis than insulin. However, at concentrations exceeding that normally found in plasma, IGF-I (100 nM) and insulin (1 μM) exerted similar effects on metabolism and mitogenesis. Although these are concentrations that disregard the effects of IGF-binding proteins on IGF-I availability, they ensure an environment that favors receptor saturation with the intention to elicit a detectable response.

The reductions in ubiquitin ligase expression are consistent with previous findings in mammalian myocyte cultures that demonstrate comparable reductions in ubiquitin ligase mRNA abundance with IGF-I and insulin treatment (35, 68). Also consistent is that atrogin-1/Fbx expression is regulated to a greater extent than MuRF1, both by IGF-I and insulin, which decrease their expression, or by compounds like wortmannin and rapamycin, which increase their expression. In mammals, atrogin-1 mRNA has a half-life of just over 1 h, whereas MuRF1 mRNA is more stable, with a half-life more than three times as long (68). This may explain why atrogin-1/Fbx transcript abundance decreases at a faster rate than MuRF1 when anabolic hormones are present. However, the 6- to 9-fold upregulation of Fbx32 compared with 1.5-fold for Fbx25 and MuRF1 after wortmannin treatment suggests that some ubiquitin ligases like Fbx25 and MuRF1 are constitutively expressed and/or are influenced by signaling independent of the PI 3-kinase/Akt pathway in rainbow trout, as they are in mammals (40). Supporting this conclusion are observations from the leucine and rapamycin treatments, in which levels of Fbx32 transcript were affected while Fbx25 and MuRF1 transcript abundance remained unchanged.

In mammals, it is clear that not all catabolic and anabolic signals have ambiguous effects on protein metabolism, rather some signals affect synthesis or degradation of specific proteins (77). For example, MuRF1 associates with myofibrillar proteins like titin, troponin, and myosin-light chain (22, 43, 89), and are implicated in the development and maintenance of cardiac and skeletal muscles. Additionally, there is some evidence involving MuRF1 in the regulation of energy metabolism because of its association with enzymes involved in ATP production (89). This suggests that MuRF1 is involved in the coordinated regulation of muscle mass and energy production, which would benefit from more stable levels of expression regulated by multiple signaling pathways, as the results in the present study suggest.

In mammals, the signaling pathway containing PI 3-kinase affects ubiquitin ligase expression via PI 3-kinase’s inactivation of FOXO transcription factors, which are involved in initiating transcription of ubiquitin ligase genes. Data presented here suggest that similar mechanisms are in place in fish. The phosphorylation and inactivation of FOXO by IGF-I and insulin were ameliorated by the PI 3-kinase inhibitor wortmannin, which also resulted in upregulation of ubiquitin ligase expression. In mammals, four FOXO proteins have been described [FOXO1, FOXO3, FOXO4, and FOXO6 (3, 50)]. In fish, FOXO5 has been identified as a homolog of the mammalian FOXO3. FOXO proteins are involved in regulation of numerous processes, including cell proliferation, nutrient metabolism, and stress resistance and are ubiquitously expressed, albeit at different levels, among tissues.

In the present experiment, the abundance of polyubiquitinated proteins is directly proportional to ubiquitin ligase expression, which supports previous proposals that ubiquitin ligases are involved in determining the rate and specificity of the ubiquitination process (33, 65). However, in mammalian myocytes, IGF-I reduced both atrogin-1 and polyubiquitin mRNA abundance (68), suggesting that the IGF-I-induced reduction in polyubiquitinated proteins is not solely an effect of reduced ubiquitin ligase abundance but of other components of the ubiquitin system as well. In mammals, reductions in basal levels of cathepsin expression (68) and cathepsin (85) and calpain activity, but not proteasome activity (37), occur in IGF-I-treated myocytes. In the current study, cathepsin expression was not reduced, which was unexpected, since IGF-I treatment is capable of inhibiting cathepsin upregulation during feed deprivation in trout (7). Differences between in vitro and in vivo conditions, or the relatively short incubation period, may be responsible for these observations. However, the absence of an effect on cathepsin expression suggests that the changes in protein degradation with IGF-I and insulin treatment are largely a result of regulation of the ubiquitin-proteasomal pathway.

Fewer components of the polyubiquitin system responded to leucine compared with hormone treatment, with Fbx32 being the only ubiquitin ligase transcript that was affected in the present study. In C2C12 myocytes, which are murine in origin, 1 and 5 mM leucine, as well as other branched-chain amino acids, reduced both atrogin-1 and MuRF1 expression (24), so the extent to which amino acids regulate expression of ubiquitin ligases may be species specific. This is logical, especially considering the differences in dietary protein requirements between species, particularly between mice and trout that require 12–20% (56) and 35–40% (55) dietary crude protein, respectively. Therefore, it is likely that differences in dietary amino acid and protein requirements would translate into differences in how dietary amino acids regulate protein turnover. The suppression of protein degradation by leucine has been demonstrated in mammals (38, 49), but this is the first
indication that this effect is present in fish. The expected stimulation of protein synthesis by leucine did not occur in the present experiment. A previous study in trout myocytes (73) found that activation of proteins downstream of TOR signaling occurs in media with amino acid concentrations between 0.2 and 0.4 mM. In the present experiment, basal media contained 0.6 mM leucine with other amino acids at similar concentrations; therefore, the stimulatory effect of amino acids on protein synthesis via TOR signaling was likely maximized before addition of the supplemental 2.5 mM leucine.

Perspectives and Significance

The ubiquitin-proteasome pathway is responsible for the majority of protein degradation during atrophy (20, 32, 36), which requires upregulation of ubiquitin ligase expression (2). However, regulation of the ubiquitin system and its contribution to protein degradation during periods of optimal growth remains less clear. Although the ubiquitination process involves several reactions with multiple enzymes included, ubiquitin ligase abundance appears to influence the rate at which protein ubiquitination and protein degradation occurs (65, 68). Therefore, maintaining low, basal levels of ubiquitin ligase expression in muscle during periods of growth and nutrient availability may be important for efficient protein accretion and optimal growth. The observed reduction in protein degradation and ubiquitin ligase expression by IGF-I, insulin, and leucine in the present study is supportive of this concept and is the first indication that this effect is present in fish. The present study also demonstrates that IGF-I, insulin, and leucine reduce ubiquitin ligase expression in fish via similar mechanisms that are observed in mammals and avians, which contribute to the anabolic nature of these compounds. Therefore, the reduction in protein degradation and resultant increase in protein accretion may be partially responsible for the correlation between plasma IGF-I and growth performance that has been observed in fish (16, 34, 45, 62).

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DISCLOSURES

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