IGF-I stimulates muscle growth by suppressing protein breakdown and expression of atrophy-related ubiquitin ligases, atrogin-1 and MuRF1

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Sacheck, Jennifer M., Akira Ohtsuka, S. Christine McLary, and Alfred L. Goldberg. IGF-I stimulates muscle growth by suppressing protein breakdown and expression of atrophy-related ubiquitin ligases, atrogin-1 and MuRF1. Am J Physiol Endocrinol Metab 287: E591–E601, 2004. First published April 20, 2004; 10.1152/ajpendo.00073.2004.—Muscle atrophy results primarily from accelerated protein degradation and is associated with increased expression of two muscle-specific ubiquitin ligases (E3s): atrogin-1 and muscle ring finger 1 (MuRF1). Glucocorticoids are essential for many types of muscle atrophy, and their effects are opposite to those of insulin-like growth factor 1 (IGF-I) and insulin, which promote growth. In myotubes, dexamethasone (Dex) inhibited growth and enhanced breakdown of long-lived cell proteins, especially myofibrillar proteins (as measured by 3-methylhistidine release), while also increasing atrogin-1 and MuRF1 mRNA. Conversely, IGF-I suppressed protein degradation and prevented the Dex-induced increase in proteolysis. IGF-I rapidly reduced atrogin-1 expression within 1 h by blocking mRNA synthesis without affecting mRNA degradation, whereas IGF-I decreased MuRF1 mRNA slowly. IGF-I and insulin also blocked Dex induction of these E3s and several other atrophy-related genes (“atrogenes”). Changes in overall proteolysis with Dex and IGF-I correlated tightly with changes in atrogin-1 mRNA content, but not with changes in MuRF1 mRNA. IGF-I activates the phosphatidylinositol 3-kinase (PI3K)-Akt pathway, and inhibition of this pathway [but not the calcineurin-nuclear factor of activated T cell (NFAT) or the MEK-ERK pathway] increased proteolysis and atrogin-1 mRNA expression. Thus an important component of growth stimulation by IGF-I, through the PI3K-Akt pathway, is its ability to rapidly suppress transcription of the atrophy-related E3 atrogin-1 and other atrogens and degradation of myofibrillar proteins.

The primary cause of the rapid loss of muscle mass and myofibrillar proteins on denervation (14, 33) or disuse (48, 51), in fasting (33), and in many systemic diseases (25) is accelerated protein breakdown. This enhancement of overall proteolysis in atrophying muscles results mainly from a general activation of the ubiquitin (Ub)-proteasome pathway (25). In various types of atrophy, including Cushing’s syndrome (22), diabetes (39), sepsis (50), cancer cachexia (2), and renal failure (41), muscles exhibit a common series of adaptations, including increased content of Ub-protein conjugates (55) and of mRNA encoding Ub (32), certain ubiquitination enzymes (26), and multiple proteasome subunits (23, 42).

Recently, we identified a number of parallel changes in gene expression that are common to these different forms of muscle atrophy (termed atrophy-related genes or “atrogenes”) (15, 23); yet it is unclear how these various transcriptional changes during atrophy are coordinately induced. The gene most dramatically induced is a new Ub ligase (E3), atrogin-1 [muscle atrophy F-box (MAFbx)], which is a muscle-specific F-box protein that is induced 8- to 40-fold in atrophy during fasting, diabetes, cancer, renal failure (15), and denervation (3, 44a; unpublished observations). In fasting, its expression increases before muscle weight loss, and its mRNA content is higher when net proteolysis is rapid (15). The strong induction of atrogin-1 must play an important role in the loss of muscle protein, because knockout mice lacking this E3 have reduced rates of denervation atrophy (3). A major goal of the present studies was to increase our understanding of the role of atrogin-1 in the atrophy process and the factors regulating its expression in atrophying and normal muscle.

Another muscle-specific E3, muscle ring finger 1 (MuRF1), is also highly induced in atrophying muscle (3). This ring finger protein was initially found in association with the myofibril (6, 31) and thus may play an important role in the breakdown of myofibrillar proteins. Knockout mice lacking MuRF1 also have a reduced capacity for muscle atrophy (3). Presumably these two E3s play critical roles in atrophy by catalyzing the ubiquitination and degradation of key muscle proteins. The present studies have investigated whether the expression of atrogin-1 and MuRF1 is regulated similarly, their possible linkage to overall rates of protein breakdown, the endocrine factors that maintain their expression at low levels in growing muscle, and the signal transduction cascade controlling their expression.

Glucocorticoids in high doses stimulate muscle proteolysis and wasting (22), and physiological levels of these steroids are required for the activation of proteolysis, accumulation of Ub conjugates, and expression of Ub mRNA during fasting, diabetes, sepsis, and uremia (34, 43, 49, 54). The synthetic glucocorticoid dexamethasone (Dex) has also been reported to promote protein breakdown and to induce atrogin-1 and MuRF1 expression in myotube cultures (3, 53) as well as in adult muscles (3). Insulin and insulin-like growth factor I (IGF-I) have anabolic effects opposite to those of adrenal steroids. Similar to its homolog insulin, IGF-I stimulates muscle protein synthesis and hypertrophy via the phosphatidylinositol 3-kinase (PI3K)-Akt pathway, and activation of this pathway can reduce muscle atrophy (44). Insulin has also been reported to rapidly reduce net protein breakdown in liver, heart, and skeletal muscle (19), but this effect has been attributed to

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IGF-1 suppresses proteolysis and ubiquitin ligase mRNA

MATERIALS AND METHODS

Cell culture. C2C12 myoblasts were obtained from American Type Culture Collection (ATCC; Manassas, VA) and grown in Dulbecco’s modified Eagle’s medium (DMEM) + 10% fetal bovine serum (ATCC). At a confluence of ~75%, myoblasts were fused by shifting medium to DMEM containing 2% horse serum (ATCC). Cells were maintained in 2% serum (differentiation medium) for 96 h. The myotubes were treated 96 h later, when myoblast fusion is complete (44), with various reagents and combinations depending on the experiment: 1 μM Dex (cycloextrim encapsulated; Sigma, St. Louis, MO) in phosphate-buffered saline (PBS), 100 nM triiodothyronine (T$_3$, Sigma) in 10 mM NaOH, R-IGF-I (Sigma; 10 ng/ml) in PBS, insulin (1 μg/ml) in PBS, 10 μM LY-29002 (Biomol, Plymouth Meeting, PA) in dimethyl sulfoxide (DMSO), rapamycin (Rap; Calbiochem, San Diego, CA; 2 ng/ml) in DMSO, 10 μM PD-98059 (Calbiochem) in DMSO, 1 μM cyclosporin (Calbiochem) in DMSO, 50 μM U-0126 and U-0124 (Calbiochem) in DMSO, and actinomycin D (Sigma; 5 μg/ml) in DMEM.

Total RNA, DNA, and protein. Total RNA was extracted from C2C12 myotube cell cultures by phenol-chloroform extraction as previously described (15). The purity of the extracted RNA was confirmed by determining the ratio of optical density at 260 nm to that at 280 nm (ratios were 1.8–2.0). The concentration of total RNA was estimated by multiplying optical density at 260 nm by 40, the molecular density of RNA. The cell protein was extracted and pelleted from the phenol-ethanol phase with use of isopropanol and quantitated by the bicinchoninic acid method (Pierce, Rockford, IL). The DNA pellet was washed and measured spectrophotometrically at 260 nm for DNA quantification (1 unit = 50 μg/ml of DNA).

Protein breakdown. To label long-lived cell proteins, C2C12 myotubes were incubated in DMEM + 2% horse serum for 72 h; then L-[3,5-3H]tyrosine (5 μCi/ml; ICN Biochemicals, Irvine, CA) was added to the medium for an additional 24 h. After they were labeled, the cells were rinsed and placed in chase medium (DMEM + 2% horse serum + 2 mM tyrosine) for 3 h to allow degradation of very-short-lived proteins. Aliquots (200 μl) of culture medium were taken at specified times for quantitation of L-[3,5-3H]tyrosine release. Proteins were precipitated at 4°C with trichloroacetic acid (TCA, 10% final concentration) and centrifuged at 15,000 rpm for 5 min. The precipitate was rinsed twice with TCA and solubilized by sonication in lysis solution (1% Triton X-100 and 1 N NaOH). Radioactivity in the TCA-soluble supernatant and the proteins (TCA-insoluble fraction) was measured using liquid scintillation counting. At the end of the chase period, cells were rinsed twice in PBS and precipitated at 4°C in 10% TCA, and the radioactivity in cell protein was measured as described above. Total radioactivity is the sum of the residual radioactivity in cell proteins and the TCA-soluble radioactivities at different time points. Protein breakdown was expressed as [%3H]tyrosine released as a percentage of total [%3H]tyrosine incorporated.

3-Methylhistidine release. Ninety-six hours after differentiation, cells were treated with Dex, IGF-I, or Dex + IGF-I and incubated for an additional 30–48 h; then the medium was collected and deproteinized with sulfosalicylic acid (5% final concentration). An aliquot of the deproteinized supernatant was applied to an ion-exchange column (8 × 40 mm, Dowex 50x-8, equilibrated with 0.2 M pyridine). The resin was washed with 0.2 M pyridine and 3-methylhistidine (3-MH) and eluted with 1.0 M pyridine. The fractions containing 3-MH were collected, dried, and reconstituted in mobile phase (15 mM sodium octane sulfonate in 20 mM KH$_2$PO$_4$). 3-MH was assayed by HPLC as previously described by Ohtsuka et al. (38). A fluorometer (model RF-535, Shimadzu; excitation at 348 nm and emission at 460 nm) was used to monitor the fluorescence derived from the reaction with ortho-phthalaldehyde.

Northern blot analysis. Total RNA was extracted from C2C12 myotube cell cultures as described above and probed for atrogin-1, MuRF1, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), met-tallothionein, and cathepsin L by Northern blot analysis as previously described (15). Atrogin-1 and poly-Ub probes were prepared as previously described (15, 32), and a DNA fragment for the MuRF1 probe was kindly provided by Dr. David Glass (Regeneron). Mouse cathepsin L (GenBank accession no. AA174215) and metallothionein 1 (GenBank accession no. NM_013602) were amplified by PCR from a mouse cDNA library in pSORT (15) and subcloned into KpnI and XbaI sites in pcDNA-A (Invitrogen) for cathepsin L and EcoRI and XbaI sites for metallothionein. Primers for PCR amplification were as follows: 5′-CGGGTACCATGATTTTTCCTTCTTCTG-3′ (forward) and 5′-TGCTCTGAAATTCACCGACAGGATAGCTGCG-3′ (reverse) for cathepsin L and 5′-CCGGAATTCATGGACCCAACTGCTCTGG-3′ (forward) and 5′-TGCTCTAGAGGCACAGCGACTTGC-3′ (reverse) for metallothionein 1. The full-length cDNAs from these vectors were used for random priming to generate probes for Northern analysis. Hybridization was performed by the method of Church and Gilbert (7) at 65°C overnight. Hybridized membranes were analyzed with a Fuji PhosphorImager and QuantityOne software (Bio-Rad). Blots were stripped and rehybridized with a mouse GAPDH probe (Ambion, Austin, TX) to ensure equivalent gel loading. In the present experiments, GAPDH mRNA was not changed by any treatment; therefore, mRNA abundance relative to GAPDH mRNA was used to normalize experiments in different lanes.

Real-time PCR. Total RNA was subjected to DNase digestion using an RNeasy kit (Qiagen, Chatsworth, CA), and total RNA purity and quantity were reassessed by measuring optical density. Total RNA (0.5 μg) was reverse transcribed using a cloned murine leukemia virus reverse transcriptase and random hexamers in a 20-μl reaction according to the manufacturer’s instructions (Applied Biosystems, Foster City, CA).

cDNA levels of atrogin-1, MuRF1, and GAPDH were measured by real-time (RT) PCR. Primers for atrogin-1 (GenBank accession no. AW051824) and MuRF1 (GenBank accession no. AY059627) were designed using Primer Express 1.5 (Applied Biosystems) and produced in an automated synthesizer according to the manufacturer’s protocol. Sequences of the primers are as follows: 5′-CAGATTTCC-CTTACTCTGATACCTCTTTG3′ (forward) and 5′-GGCCAGAGC- TCTGAA-3′ (reverse) for mouse atrogin-1 and 5′-AGACAACC-CTCTGTCCTACAG-3′ (forward) and 5′-ACAACTGTTGGCG-
CAAGTG-3′ (reverse) for mouse MuRF1. To normalize target cDNA values, GAPDH cDNA levels were quantified using commercially available rodent GAPDH primers (Applied Biosystems).

SYBR green. RT-PCR was performed on a LightCycler System (Roche Diagnostics, Nutley, NJ) with the following parameters: denaturation at 95°C for 10 min followed by 40 cycles of 95°C for 10 s and 55°C for 5 s for annealing and 72°C for 12 s for extension. For each gene, first-strand cDNAs were amplified utilizing the Fast-Start DNA Master SYBR green kit and protocol (Roche). Briefly, each 20-μl reaction contained 2 μl of FastStart reaction mix with enzyme, 3 mM MgCl₂, target cDNA specific primers (0.25 μM each), and 4 ng of first-strand cDNA sample. Each sample was run in duplicate, and all runs included a negative control. The gene of interest was quantified relative to a standard curve of the cDNA of that gene (atrogin-1, MuRF1, or GAPDH), which also served as the positive control.

Statistical analysis. Results for individual experiments were replicated in two to six independent experiments and analyzed using the General Linear Model procedure of the Statistical Analysis System software package (release 6.09, SAS Institute, Cary, NC) or SPSS statistical package (version 11.0, SPSS, Chicago, IL) and presented as means ± SD or means ± SE. Statistical significance was set at *P < 0.05.*

RESULTS

Effects of Dex and IGF-I on protein breakdown. To determine whether treatment of myotubes with the synthetic glucocorticoid Dex could serve as a useful in vitro model of atrophy, we treated C₂C₁₂ cells with Dex and/or T₃ for 18 h and followed the degradation of long-lived proteins. In adult animals, glucocorticoids enhance proteolysis in muscle, especially myofibrillar proteins (27), and thyroid hormones have been reported to enhance protein breakdown in conjunction with glucocorticoids in rat muscle (1). T₃ was therefore added with Dex to test for any synergistic effects on protein breakdown and to better simulate in vivo conditions. After cell proteins were labeled for 24 h with L-[3,5-³H]tyrosine, its breakdown was measured (28). Because it cannot be reutilized in new protein synthesis and is not metabolized in muscle, 3-MH can be used to follow the degradation of actin. The standard method used for the 3-MH assay in vivo has been adapted for use in cell culture (38).

ACKNOWLEDGMENTS

We thank lines from the M. M. Dessler laboratory, as well as T. W. McEwen, for providing the rat muscle culture. We also thank C. M. L. P. for providing the rat muscle culture. We also thank C. M. L. P. for providing the rat muscle culture. We also thank C. M. L. P. for providing the rat muscle culture.
and Dex + IGF-I for 18 h on these parameters (Table 1). Total RNA decreased by 15% in the Dex-treated group \((P < 0.01)\) but increased by 17% in the IGF-I-treated and IGF-I + Dex-treated groups \((P < 0.01)\). By contrast, the total DNA content did not change in any condition, which is consistent with no change in cell number and cell fusion. Even in this short period, after treatment with Dex alone, total protein in the cultured cells decreased by \(-10\%\) \((P < 0.05)\), whereas if IGF-I was also present, total protein increased by \(-20\%\) \((P < 0.01)\). Interestingly, the increase in the group treated with IGF-I alone was smaller and failed to reach significance within 18 h. [At this time, there should be little effect on protein accumulation through a 20% decrease in degradation of long-lived proteins (Fig. 1).]

These increases in RNA and protein by IGF-I indicate a stimulation of growth, whereas the decreases in these parameters after Dex suggest an atrophy of myotubes. In related studies, Dex administration for a longer period (2 days) caused a clear decrease in fiber diameter (45). Most importantly, when IGF-I was present with Dex, there was a clear increase in cell...
Table 1. Dex decreases total myotube content of RNA and protein, while IGF-I promotes these processes and blocks effects of Dex

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Dex</th>
<th>IGF-I</th>
<th>Dex + IGF-I</th>
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<tbody>
<tr>
<td>Total RNA (ng/g)</td>
<td>100±7</td>
<td>85±7†</td>
<td>120±4‡</td>
<td>116±7†</td>
</tr>
<tr>
<td>Total protein (ng/g)</td>
<td>100±7</td>
<td>90±7*</td>
<td>110±11§</td>
<td>120±9‡</td>
</tr>
<tr>
<td>Total DNA (ng/g)</td>
<td>100±13</td>
<td>108±22</td>
<td>120±26</td>
<td>116±27</td>
</tr>
</tbody>
</table>

Values (means ± SD) were quantitated as mg/plate and are expressed as percentage of control (n = 4). Samples were analyzed 18 h after treatment with dexamethasone (Dex) and/or insulin-like growth factor (IGF-I). *P ≤ 0.05; †P < 0.01 vs. control. §P < 0.01 vs. Dex.

protein and RNA. In other words, the addition of IGF-I completely reversed the catabolic effects of Dex, as was also seen with measurements of overall proteolysis, degradation of myofibrillar components (Fig. 1), and atrogin-1 expression (see below).

Atrogin-1 and poly-Ub expression. To determine how Dex or IGF-I influenced expression of atrophy-related genes, Northern blot analysis and RT-PCR analysis were performed after treatment of myotubes with Dex or IGF-I. By Northern analysis, atrogin-1 mRNA rose significantly within 2 h after Dex administration (P < 0.05; Fig. 2). Maximum induction of 2.5-fold was evident by 6 h and remained at this level through 24 h of treatment (P < 0.001). However, using RT-PCR analysis, a more sensitive method for quantitation of gene expression, we found a fivefold increase in atrogin-1 expression after Dex treatment (see Fig. 4A; P < 0.001). Hence, after Dex treatment, atrogin-1 expression appeared to be maximally elevated before the increases in protein breakdown and remained elevated when protein breakdown was accelerated, as was found in vivo (15).

Poly-Ub mRNA has been used as a convenient and sensitive marker for general activation of the Ub-proteasome pathway in atrophying muscles. In the myotubes, poly-Ub mRNA also significantly increased within 2 h (Fig. 2; P < 0.05), but only by 26%, and then remained at this level. Because atrogin-1 mRNA is increased much more than poly-Ub during muscle atrophy in vivo (23) as well as in myotubes, atrogin-1 is clearly a more sensitive marker for activation of the atrophy process, and it correlates with an overall increase in proteolysis (see below).

On the other hand, IGF-I and insulin reduce levels of atrogin-1 mRNA in normal cells (Figs. 3A and 4A). These effects occurred rapidly; for example, IGF-I or insulin decreased atrogin-1 mRNA by 22% within 30 min and by 70% at 6 h (P < 0.001; Fig. 3A). In addition, IGF-I was able to quickly decrease both transcripts of poly-Ub mRNA (2.4 and 1.3 kb), although the suppression of the 2.4-kb transcript was twice as great as that of the 1.3-kb transcript (P < 0.01; Fig. 3B).

In vivo, insulin inhibits many of the catabolic effects of glucocorticoids (54), and in C2C12 cells, when Dex and IGF-I or insulin were added to the medium simultaneously, IGF-I or insulin blocked the increase in atrogin-1 expression by Dex (P < 0.05; Fig. 4A). Furthermore, after pretreatment with Dex for 6 h to maximally induce atrogin-1, the addition of IGF-I or insulin in the presence of Dex was able to reverse the increase in atrogin-1 expression (P < 0.05; Fig. 4B). Thus the IGF-I effects are clearly dominant over the ability of glucocorticoids to induce atrogin-1 and to stimulate proteolysis (Fig. 1).

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**Fig. 2.** Treatment of myotubes with Dex rapidly increases atrogin-1 and polyubiquitin (poly-Ub) mRNA. Myotubes (n = 4/group) were treated with Dex (400 ng/ml), subjected to Northern analysis (10 μg of total RNA loaded per lane), and probed with atrogin-1, poly-Ub, and GAPDH.

**Fig. 3.** IGF-I rapidly suppresses atrogin-1 and poly-Ub mRNA in C2C12 myotubes. A: myotubes were treated with IGF-I (10 ng/ml) for 3 h. cDNA levels of mouse atrogin-1 and GAPDH were assayed by real-time (RT) PCR. IGF-I decreased atrogin-1 mRNA 22% within 30 min (P < 0.05) and by 70% within 3 h (P < 0.001). B: total RNA from control and IGF-I-treated myotubes was probed and quantitated by Northern analysis (10 μg of total RNA loaded per lane) for poly-Ub mRNA. PolyUbA, 2.4-kb transcript; polyUbB, 1.3-kb transcript; polyUbA+B, average of both transcripts. Values are expressed relative to control (n = 4/group), and results were reproduced in ≥3 independent experiments.
IGF-I suppresses proteolysis and ubiquitin ligase mRNA

**Fig. 4.** IGF-I and insulin block Dex-induced atrogin-1 and MuRF1 expression in C2C12 myotubes. A: RT-PCR analysis of atrogin-1 and MuRF1 expression after 6 h of treatment with Dex [400 ng/ml, T3 (100 ng/ml)] and/or IGF-I (10 ng/ml) or insulin (1 µg/ml). B: RT-PCR analysis of atrogin-1 expression after 6 h of treatment with Dex followed by IGF-I (10 ng/ml) or insulin (1 µg/ml) for an additional 6 h. Addition of IGF-I or insulin to myotubes after 6 h of treatment with Dex did not reverse Dex-induced increase in MuRF1 expression (data not shown). C: RT-PCR analysis demonstrating rapidity and magnitude of suppression of atrogin-1 and MuRF1 mRNA in C2C12 myotubes after treatment with IGF-I (10 ng/ml). Values are means ± SE (n = 4–5/group).

**A** and **B**: Mean expression ± SE after 6 h of Dex treatment with IGF-I (10 ng/ml) or insulin (1 µg/ml) or Dex and IGF-I (10 ng/ml) or insulin (1 µg/ml) for an additional 6 h on control, Dex, IGF-I, and insulin. 

**C**: Mean expression ± SE after addition of IGF-I (10 ng/ml) or insulin (1 µg/ml) to C2C12 myotubes after treatment with IGF-I (10 ng/ml). Values are means ± SE (n = 4–5/group).

*P ≤ 0.05 vs. control. **P ≤ 0.01; ***P ≤ 0.001 vs. Dex.

**MuRF1 expression.** The other important E3 induced during atrophy, MuRF1, was also induced by Dex, but to a much lesser extent than atrogin-1 (Fig. 4A). RT-PCR analysis revealed a twofold increase in MuRF1 expression after Dex treatment (P < 0.05), which is significantly smaller than the fivefold increase observed in atrogin-1 mRNA by this approach (P < 0.01). After addition of IGF-I or insulin, MuRF1 mRNA also decreased, but not as quickly or dramatically as atrogin-1 mRNA in the same cells. For example, atrogin-1 mRNA decreased by 60% within 2 h (P < 0.01), whereas MuRF1 mRNA decreased by only 36% within 18 h (P < 0.06; Fig. 4, A and C). Furthermore, IGF-I was able to block the Dex-induced stimulation of MuRF1 expression and to maintain it at control levels in the presence of Dex [96 ± 9% (mean ± SE) of control, P < 0.05] but did not reduce mRNA well below control levels, as was seen with atrogin-1 mRNA. Thus expression of atrogin-1 and MuRF1 is increased by Dex and suppressed by IGF-I, although atrogin-1 mRNA is much more responsive to these hormonal signals. **IGF-I blocks atrogin-1 mRNA synthesis without affecting its degradation.** We tested whether this rapid decrease in atrogin-1 mRNA content by IGF-I or insulin was due simply to inhibition of gene transcription or whether it also involved accelerated mRNA destruction. To evaluate mRNA stability, myotubes were treated with an inhibitor of transcription, actinomycin D, IGF-I, or actinomycin D + IGF-I. Atrogin-1 mRNA decreased by one-half within 1 h of actinomycin D treatment and by 74% by 3 h (Fig. 5; P < 0.001). These effects of actinomycin D closely paralleled the changes in atrogin-1 mRNA seen after IGF-I treatment and indicate a short half-life of this mRNA. Moreover, when IGF-I and actinomycin D were added simultaneously to the cell cultures, the fall in atrogin-1 was similar to the changes seen with IGF-I treatment alone (Fig. 5). Therefore, the effects of IGF-I on mRNA levels can be explained simply by blocking transcription, and evidence on the underlying mechanism is reported elsewhere (45). Furthermore, IGF-I + actinomycin D did not have a greater effect on atrogin-1 mRNA decay than IGF-I or actinomycin D alone; so IGF-I does not appear to promote mRNA breakdown.

By contrast, MuRF1 mRNA did not fall significantly within 3 h after the addition of actinomycin D, even when IGF-I and actinomycin D were present together (Fig. 5). This stability of MuRF1 mRNA can explain why the effects of IGF-I on its expression are much slower and less dramatic than those on atrogin-1. Thus, the ability of IGF-I to quickly decrease atrogin-1 mRNA levels appears to be due to IGF-I blocking the transcription of this gene and exposing the short half-life of atrogin-1 mRNA, whereas MuRF1 mRNA, being quite stable, responds slowly to an inhibition of transcription.

**Expression of other atrogenes.** Recently, our laboratory identified a set of genes whose transcription rises or falls in various types of muscle atrophy (20, 23), including atrogin-1, MuRF1, and poly-Ub. To test whether other genes induced in atrophy are regulated similarly by glucocorticoids and IGF-I, we measured the levels of several other mRNAs that were highly upregulated during atrophy (23) (Table 2). Metallothionein, which is induced by oxidative stress and heavy metals (37), is among the genes most highly induced in atrophying muscles (23). The cysteine protease cathepsin L is the lysosomal protease that is upregulated in various types of atrophy (23). As found with atrogin-1, MuRF1, and poly-Ub,
mRNA of these atrogenes was increased (as shown by Northern analysis) after Dex treatment, and their expression decreased similarly with IGF-I addition. On the basis of these examples, when the atrophy-related transcriptional program is activated by Dex treatment, IGF-I is able to reverse or block the expression of many atrogenes, including metallothionein and cathepsin L (although a more systematic transcriptional array analysis is necessary to test whether all atrogenes are regulated in this manner).

Atrogin-1 mRNA correlates with protein breakdown. To examine the possible connection between changes in the expression of these E3s and protein breakdown, we tested whether levels of atrogin-1 mRNA expression correlated with the overall rate of muscle protein breakdown after treatment with IGF-I or Dex (Fig. 6). A highly positive linear correlation ($R^2 = 0.79, P < 0.01$) was found between the rate of protein breakdown and atrogin-1 mRNA levels (by Northern blot analysis) after treatment with IGF-I or Dex. This correlation was sharpest when atrogin-1 mRNA content was <200% of control. When atrogin-1 expression increased further, greater scatter was found, perhaps because other factors may be influencing this relationship under these conditions. No similar correlation was seen between overall rates of protein breakdown and content of MuRF1 mRNA ($R^2 = 0.14, P > 0.10$). The tight association between atrogin-1 and protein breakdown further argues that atrogin-1 is a critical E3 during the atrophy process.

Signal transduction and atrogin-1 expression. Because atrogin-1 appears to play a key role in the atrophy process, it is important to decipher which signal transduction pathways mediate its repression by IGF-I and how IGF-I blocks its induction by Dex. IGF-I and insulin are known to promote muscle hypertrophy via the PI3K-Akt pathway (44). We therefore tested whether inhibition of this growth-promoting pathway may lead to a stimulation of atrogin-1 expression and protein breakdown (Fig. 7A). Inhibiting PI3K with LY-294002 or mammalian target of Rap (mTOR) with Rap for 6 h increased atrogin-1 mRNA levels by approximately twofold as determined by Northern and RT-PCR analysis ($P < 0.01$). On the other hand, MuRF1 expression was not significantly elevated after treatment with either inhibitor as determined by Northern analysis and only slightly increased when the more sensitive RT-PCR analysis was used to evaluate Rap treatment only $[+9.3 \pm 6\%$ for LY-29002 and $+20 \pm 5\%$ for Rap (means ± SE)]. Also, the addition of LY-29002 ($P < 0.001$) and, to a lesser extent, Rap ($P < 0.01$) for 18 h (with initial

Table 2. Dex increases expression of atrophy-related genes (“atrogenes”) in C2C12 myotubes, while IGF-I suppresses their expression and blocks effects of Dex

<table>
<thead>
<tr>
<th>Atrogene</th>
<th>Dex</th>
<th>IGF-I</th>
<th>Dex + IGF-I</th>
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<tbody>
<tr>
<td>Atrogin-1</td>
<td>530^a</td>
<td>30^a</td>
<td>35^d</td>
</tr>
<tr>
<td>MuRF1</td>
<td>210^b</td>
<td>64^b</td>
<td>101^c</td>
</tr>
<tr>
<td>Poly-Ub</td>
<td>143^a</td>
<td>58^a</td>
<td>117^c</td>
</tr>
<tr>
<td>Metallothionein</td>
<td>463^b</td>
<td>74^b</td>
<td>112^d</td>
</tr>
<tr>
<td>Cathepsin L</td>
<td>172^a</td>
<td>83^a</td>
<td>108^c</td>
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Values, expressed as percentage of untreated control, were quantitated by Northern analyses (except atrogin-1 and MuRF1, which were analyzed by RT-PCR) and are average values from 2–6 experiments per gene. MuRF1, muscle ring finger 1; poly-Ub, polyubiquitin. ^P < 0.05; ^P < 0.01 vs. control. ^P < 0.05; ^P < 0.01 vs. control.
effects observed within 6 h) increased the rate of breakdown of long-lived proteins in cultured myotubes (Fig. 7B).

IGF-I also activates the MEK-ERK pathway, and glucocorticoid induction of Ub mRNA is transcriptionally regulated by MEK1 (30), although the potential importance of this pathway in skeletal muscle hypertrophy is uncertain (11). Activation of the calcineurin-nuclear factor of activated T cell (NFAT) pathway has also been implicated as a mediator of cardiac hypertrophy (36), but its role in skeletal muscle is less clear (11, 46). To test whether these pathways are involved in the regulation of atrogin-1 expression, we incubated cultured myotubes with inhibitors of the MEK1/2 pathway (U-0126 and PD-98059) and the calcineurin-NFAT pathway (cyclosporin). Inhibition of these pathways for 18 h resulted in no change in atrogin-1 mRNA content as determined by RT-PCR (Fig. 7C). Thus these pathways do not appear to influence atrogin-1 expression, which appears to be regulated by the PI3K-Akt pathway. Extensive evidence for this conclusion and for involvement of the forkhead (Foxo) family of transcription factors is reported elsewhere (45).

**DISCUSSION**

During atrophy, skeletal muscles exhibit a common set of biochemical and transcriptional changes that constitute an "atrophy program" (20, 23). Here we have demonstrated that glucocorticoids, which are essential for many of these characteristic changes seen in several types of atrophy in vivo (34, 43, 49, 54), induce similar changes in cultured myotubes. Dex in conjunction with T3 significantly increases degradation of the bulk of cell proteins in the myotubes, including breakdown of myofibrillar proteins. These changes were accompanied by a
reduction in total protein and RNA content (i.e., ribosomes) within only 18 h and a decrease in cell diameter by 2 days (45). Although the Dex-induced increase in overall proteolysis in culture appears small, such a 22% increase in overall degradation rates can have a large effect on total muscle protein if sustained over several days or weeks. Moreover, in these same cells, the rate of breakdown of myofibrillar proteins, primarily actin, increased \textasciitilde 100%, as shown by 3-MH production. In myotubes, myofibrillar proteins typically comprise only 1–2% of the total cell protein, whereas in adult muscle they comprise 60–70%. Thus, such an acceleration of proteolysis should have major effects on muscle size and functional capacity.

Conversely, IGF-I decreased basal levels of protein breakdown, increased total RNA, and, most notably, prevented the Dex-induced increase in overall and myofibrillar protein breakdown and the decrease in protein content. Thus IGF-I is a dominant signal over glucocorticoids in determination of muscle protein balance in vitro, as has been suggested for insulin in vivo (17). Simultaneous administration of IGF-I with glucocorticoids to rats significantly attenuates the glucocorticoid-induced muscle atrophy and myofibrillar protein breakdown as measured by 3-MH excretion (21). Accordingly, only when insulin is low (such as during fasting) are the catabolic effects of glucocorticoids on muscle evident (54). Thus, maintaining high levels of insulin and/or IGF-I when glucocorticoids are in excess could potentially have therapeutic benefits. However, IGF-I was not found to prevent the sepsis-induced increase in total and myofibrillar protein breakdown (8), perhaps because of the development of insulin resistance. The insulin resistance that is commonly seen in sepsis and certain types of cancer (28, 29) is probably due to the action of TNF-\(\alpha\), which has also been proposed to stimulate muscle atrophy and may reduce the capacity of IGF-I or insulin to suppress proteolysis (40). Other proinflammatory cytokines and eicosanoids may also play an important role in the etiology of muscle wasting (52), and their effects on atrogin-1 expression and proteolysis warrant careful investigation.

Dex markedly increases the expression of the critical E3s atrogin-1 and MuRF1 (but to a lesser extent) and the other atrophy-related genes, poly-Ub, metallothionein, and cathepsin L. The increase in cathepsin L also suggests that another proteolytic system, besides the Ub-proteasomal pathway, is important, whereas metallothionein, the function of which in atrophy is unknown, curiously showed the largest increase in mRNA after Dex treatment and large increases in vivo during various types of atrophy (23). Poly-Ub mRNA, the expression of which has served as the benchmark for activation of Ub-mediated proteolysis in atrophying muscles (55), also increased after Dex administration but to only one-third of the extent of atrogin-1 mRNA. Furthermore, recent studies during multiple types of muscle wasting (23), sepsis (56), and denervation or disuse atrophy (44a; unpublished observations) demonstrated much greater changes in E3 expression (e.g., a 40-fold increase in atrogin-1 and a 15-fold increase in MuRF1). Clearly, mRNAs for these E3s are the most sensitive markers for atrophy.

In contrast to Dex, IGF-I rapidly suppresses atrogin-1 and poly-Ub expression and also suppresses MuRF1, along with several of the newly identified atrogenes. The Dex-induced induction and the IGF-I suppression of these genes further demonstrate a coordinate regulation of their expression. However, the reduction of atrogin-1 mRNA is especially rapid after IGF-I administration, whereas MuRF1 expression falls only slowly. Because the suppression of atrogin-1 mRNA by IGF-I or insulin is very rapid, this effect is probably a critical early component of their growth-promoting actions. Although IGF-I given together with Dex prevented MuRF1 induction, IGF-I treatment after Dex administration was not able to decrease MuRF1 expression rapidly, as occurred with atrogin-1. Nevertheless, the present findings indicate a common regulatory mechanism. Experiments with actinomycin D demonstrate that atrogin-1 mRNA is quite short-lived (half-life \textasciitilde 1 h), whereas MuRF1 mRNA is quite long-lived. Moreover, because the decay of atrogin-1 mRNA after blocking transcription was similar with and without IGF-I and resembled that when IGF-I alone was present, the IGF-I effects appear to be due to its blocking mRNA production, without any effect on mRNA degradation rate. Related studies from this laboratory demonstrate that IGF-I and insulin block atrogin-1 expression by inactivating the transcription factors Foxo 1 and 3, which act on the atrogin-1 promoter (45). Moreover, after these hormonal treatments, MuRF1 and atrogin-1 mRNA change in similar directions, although changes in MuRF1 mRNA are slower and less dramatic. This lack of responsiveness of MuRF1 thus reflects the inherent stability of its mRNA.

Moreover, because the overall rate of protein breakdown correlated strongly with the content of atrogin-1 mRNA (but surprisingly not with that of MuRF1 mRNA), it is very likely that the atrogin-1 function as an E3 is tightly linked to protein breakdown and especially to the degradation of myofibrillar proteins. Perhaps atrogin-1 directly catalyzes the breakdown of myofibrillar proteins or influences this process indirectly (e.g., by promoting degradation of proteins that regulate this process); alternatively, atrogin-1 transcription may be coordinately regulated with the control of proteolysis. This failure of changes of MuRF1 expression to correlate with overall proteolysis is surprising, because MuRF1 is directly associated with the myofibril (6). In addition to these two E3s, it is likely that other proteolytic processes are activated in atrophying muscle cells and that coordinated activation of these pathways is necessary for the loss of muscle mass (24). For example, IGF-I and insulin retard lysosomal autophagic vacuole formation in muscle cells and other cells (12, 19); however, here we show for the first time that they also inhibit breakdown of contractile proteins, which is a nonlysosomal process (47).

The PI3K-Akt pathway is necessary for muscle hypertrophy (4, 44), and this pathway is activated in response to insulin, IGF-I, and exercise (3, 5). Recently, IGF-I-induced muscle hypertrophy was shown to depend on activation of PI3K, Akt, and mTOR (4, 44), but the effect was attributed solely to the general activation of protein synthesis (10). The present study is the first to demonstrate that this growth stimulation also involves suppression of proteolysis and atrophy genes. IGF-I also activates the Ras-Raf-MEK-ERK pathway (35), and some authors have proposed that the calcineurin-NFAT signaling pathway influences fiber type, as well as muscle fiber hypertrophy (36, 46). However, inhibition of MEK1/2, MAPK, or calcineurin resulted in no change in atrogin-1 mRNA levels, whereas inhibition of PI3K or mTOR increased atrogin-1 expression. In addition, inhibition of PI3K by LY-29002 increased the rate of protein breakdown (as did inhibition of mTOR by Rap, although to a lesser extent). These findings...
indicate that atrogin-1 is a regulated downstream target of PI3K and that inhibition of atrogin-1 expression occurs during hypertrophy induced by IGF-I and exercise. Elsewhere we present evidence that IGF-I blocks atrogin-1 expression through PI3K and stimulation of Akt phosphorylation and inactivation of the Foxo family of transcription factors (45). However, the mechanism by which inhibition of mTOR increases atrogin-1 expression and proteolysis remains to be determined. In any case, this demonstration of a key role of the IGF-I-PI3K-Akt pathway in the suppression of atrogin-1 expression and muscle proteolysis can account for the ability of this pathway to retard denervation atrophy (4) and muscle wasting in starvation and diabetes (15). These findings together indicate an important new mechanism contributing to the stimulation of growth by IGF-I, insulin, and exercise, by reducing degradation of contractile proteins, rapidly suppressing the transcription of atrogin-1 and other key atrogenes, and thus blocking the atrophy program. These experiments, together with our related studies implicating the Foxo family of transcription factors in the atrophy process (45), suggest that muscle hypertrophy and atrophy involve opposite changes in the same signal transduction pathway and, consequently, opposite changes in the patterns of gene expression, protein translation, and degradation. Significant gaps remain in our present understanding, and it will be important for future studies to identify the proteins that are ubiquitinated by atrogin-1, leading to muscle atrophy, to decipher which atrogenes are coordinate controlled, and to elucidate how the PI3K-Akt-Foxo pathway is altered in specific conditions associated with muscle wasting.

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