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

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Running title: IGF-1 suppresses proteolysis and ubiquitin-ligase mRNA
ASTRACT

Muscle atrophy results primarily from accelerated protein degradation and is associated with increased expression of two muscle-specific ubiquitin-ligases, atrogin-1 and MuRF1. Glucocorticoids are essential for many types of muscle atrophy, and their effects are opposite to IGF-1 and insulin, which promote growth. In myotubes, dexamethasone inhibited growth and enhanced the breakdown of long-lived cell proteins, especially of myofibrillar proteins (as measured by 3-methylhistidine release), while also increasing atrogin-1 and MuRF1 mRNA. Conversely, IGF-1 suppressed protein degradation and prevented the dexamethasone-induced increase in proteolysis. IGF-1 rapidly reduced atrogin-1 expression within one hour by blocking mRNA synthesis without affecting mRNA degradation, while IGF-1 decreased MuRF1 mRNA slowly. IGF-1 and insulin also blocked the dexamethasone induction of these ubiquitin-ligases and several other atrophy-related genes ("atrogenes"). The changes in overall proteolysis with dexamethasone and IGF-1 correlated tightly with the changes in atrogin-1 mRNA content, but not with the changes in MuRF1 mRNA. IGF-1 activates the PI3-kinase/Akt pathway and inhibition of this pathway (but not the calcineurin/NFAT or MEK/ERK pathways) increased proteolysis and atrogin-1 mRNA expression. Thus, an important component of growth stimulation by IGF-1, through the PI3-kinase/Akt pathway, is its ability to rapidly suppress transcription of the atrophy-related E3, atrogin-1, other atrogenes, and degradation of myofibrillar proteins.

Keywords: glucocorticoids, 3-methylhistidine, insulin, PI3-kinase/Akt pathway
INTRODUCTION

The primary cause of the rapid loss of muscle mass and myofibrillar proteins upon 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 have 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 (MAFbx), which is a muscle-specific F-box protein that is induced 8-40 fold in atrophy during fasting, diabetes, cancer, renal failure (15), and denervation (J.M. Sacheck, unpublished observations)² (3). In fasting, its expression increases prior to muscle weight loss, and its mRNA content is high when net proteolysis is rapid (15). The strong induction of atrogin-1 must play an important role in the loss of muscle protein since
knock-out mice lacking this Ub-ligase 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, 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. Knock-out 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 are 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). Both insulin and insulin-like growth factor 1 (IGF-1) have anabolic effects opposite to those of
adrenal steroids. Like its homolog insulin, IGF-1 stimulates muscle protein synthesis and hypertrophy via the phosphotidyl-inositol-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 suppressing lysosomal proteolysis (9). In fact, several studies have failed to demonstrate effects of insulin on degradation of the major constituents of muscle, i.e., myofibrillar proteins (16), which are degraded by the Ub-proteasome pathway (47).

The present experiments have used myotube cultures to learn more about the regulation of atrogin-1 and MuRF1 expression and protein degradation during muscle atrophy and to test whether the growth factors, IGF-1 and insulin, can block the stimulation of proteolysis and the expression of these E3s. Early studies had suggested that hypertrophy of muscle may involve a suppression of overall protein degradation (13), but this possibility has not been reinvestigated in light of recent advances in our understanding of the degradative process. Evidence is presented here that synthesis of atrogin-1 mRNA is rapidly suppressed by IGF-1 and insulin through the PI3K/Akt pathway and that expression of atrogin-1 (but not of MuRF1) is coordinately regulated with overall protein breakdown, especially the degradation of myofibrillar proteins. Related studies on the signaling system by which IGF-1 suppresses atrogin-1 transcription will appear elsewhere (45). These findings together help
clarify the molecular mechanisms of the muscle wasting in diverse disease states where insulin levels fall or where there is insulin resistance.

EXPERIMENTAL PROCEDURES

Cell Culture. C2C12 myoblasts were obtained from American Type Culture Collection (ATCC, Manassas, VA) and grown in Dulbecco’s modified eagle media (DMEM) + 10% fetal bovine serum (ATCC). At a confluence of approximately 75%, myotubes were fused by shifting media to DMEM containing 2% horse serum (ATCC). Cells were maintained in 2% serum (differentiation media) for 96 hr. The myotubes were treated 96 hr later, when myoblast fusion is complete (44), with various reagents and combinations depending on the experiment: dexamethasone (cyclodextrin encapsulated, Sigma, St. Louis, MO) at 1 µM in phosphate-buffered saline (PBS), triiodothyronine (Sigma) at 100 nM (in 10 mM sodium hydroxide), R3-IGF-1 (Sigma) at 10 ng/ml in PBS, insulin at 1 µg/ml in PBS, LY29002 (Biomol, Plymouth Meeting, PA) at 10 µM in dimethyl sulfoxide (DMSO), rapamycin (Calbiochem, San Diego, CA) at 2 ng/ml in DMSO, PD98059 (Calbiochem) at 10 µM in DMSO, cyclosporin (Calbiochem) at 1 µM in DMSO, U0126 and U0124 (Calbiochem) at 50 µM in DMSO, and actinomycin D (Sigma) at 5 µg/ml in DMSO.

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 and 280 nm (ratios were between 1.8 and 2.0). The concentration of total RNA was estimated by multiplying optical density at 260 nm by 40, molecular density of RNA. The cell protein was extracted and pelleted from the phenol/ethanol phase using isopropanol and quantitated by the BCA method (Pierce, Rockford, IL). The DNA pellet was washed and measured spectrophotometrically at 260 nm for DNA quantification (one unit equals 50 μg/ml of DNA).

**Protein Breakdown.** To label long-lived cell proteins, C2C12 myotubes were maintained in DMEM + 2% horse serum for 72 hr and then 5 μCi of L- [3,5-3H]tyrosine per ml (ICN Biochemicals, Inc., Irvine, CA) was added to the media for an additional 24 hr. After labeling, cells were rinsed and placed in chase medium (DMEM + 2% horse serum + 2 mM tyrosine) for 3 hr 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 sonicating in lysis solution (1% Triton X-100 and 1N sodium hydroxide). Radioactivity in the TCA-soluble supernatant and the proteins (TCA-insoluble fraction) were measured using liquid scintillation counting. At the end of chase period, cells were rinsed twice
in PBS and precipitated at 4°C in 10% TCA, and the radioactivity in cell protein 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 the L-[3,5-3H]tyrosine released as a percentage of total L- [3,5-3H]tyrosine incorporated.

3-Methylhistidine Release. After treatment with Dex, IGF-1, or Dex plus IGF-1 at 96 hr after switching to differentiation media, cells were incubated for 30-48 hr after which media was collected and de-proteinized with sulfosalicylic acid (5% final concentration). An aliquot of the de-proteinized supernatant was applied onto an ion exchange column (8x40 mm, Dower 50x-8, equilibrated with 0.2 M pyridine). After washing the resin with 0.2 M pyridine, 3-methylhistidine (3-MH) was 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 KH₂PO₄). 3-MH was assayed by HPLC method previously described by Ohtsuka et al. (38). A Shimadzu RF-535 fluorometer (EX: 348 nm and EM: 460 nm) was used to monitor the fluorescence derived from the reaction with ortho-phthalaldehyde.

Northern Blot Analysis. Total RNA was extracted from C2C12 myotubes cell cultures as described above and probed for atrogin-1, MuRF1, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), metallothionein, 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 muscle
RING Finger 1 (MuRF1) probe was kindly provided by Dr. David Glass (Regeneron, Inc.). Mouse cathepsin L and metallothionein 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. Forward and reverse primers for PCR amplification were as follows:
cathepsin L: 5’-CGGGGTACCATGAATCTTTACTCCTTTTGGCT-3’, 5’TGCTCTAGAAATTCAGACAGGATAGCTGGC-3’ and metallothionein: 1 5’-CCGGAATTCATGGACCCAACTGCTCCTGC-3’, 5’-TGCTCTAGAGGCACAGCACGTGCACTTGTC-3’. 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 at 65°C overnight (7). Hybridized membranes were analyzed by using a Fuji Phosphorimager with 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, and therefore mRNA abundance relative to GAPDH mRNA was used to normalize mRNAs in different lanes.

Real-time PCR. Total RNA was subjected to DNase digestion using an RNeasy kit (QIAGEN Inc., Chatsworth, CA) and total RNA purity and quantity were re-assessed 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 manufacturer’s instructions (Applied
cDNA levels of atrogin-1, MuRF1, and GAPDH were assessed by real-time (RT)-PCR. Primers for atrogin-1\textsuperscript{5} and MuRF1\textsuperscript{6} were designed using the Primer Express™ 1.5 Program (Applied Biosystems) and produced in an automated synthesizer according to the manufacturer’s protocol. Sequences of the forward primers and reverse primers are as follows: mouse atrogin-1: 5'-CAGATTCTCCTACTGTATACCTCCTTGT-3' – 5'GGC GGACGGCTG AA-3', mouse MuRF1: 5'-AGGACA ACCTCGTGCCTACAAG-3'– 5'-ACA ACCTGTGCCGCAAGTG-3'. 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 the LightCycler instrument (Roche Diagnostics, Nutley, NJ) using the following cycle parameters: denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 10 sec and 55°C for 5 sec for annealing and 72°C for 12 sec for extension. For each gene, first-strand cDNAs were amplified utilizing the FastStart DNA Master SYBR Green kit and protocol (Roche). Briefly each 20 µL reaction contained 2 µL FastStart Reaction Mix with enzyme, 3 mM MgCl\textsubscript{2}, target cDNA specific primers (0.25 µmol each), and 4 ng of first-strand cDNA sample. Each sample was run in duplicate and all runs included a negative control. Quantification of the gene of interest was done 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 2-6 independent experiments and analyzed using the General Linear Model procedure of the Statistical Analysis System (SAS) software package (Release 6.09, SAS Institute Inc., Cary, NC) or SPSS statistical package, Version 11.0 (SPSS Inc., Chicago, IL) and presented as either means ± standard deviation (SD) or means ± standard error (SE) as indicated. Statistical significance was set at $P<0.05$.

**RESULTS**

**Effects of dexamethasone and IGF-1 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 triiodothyronine (T3) for 18 hr and followed the degradation of long-lived proteins. In adult animals, glucocorticoids enhance proteolysis in muscle, especially of 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 in order to test for any synergistic effects on protein breakdown and to better simulate *in vivo* conditions. After labeling cell proteins for 24 hr with L-[3,5-3H]tyrosine, its release from cell proteins was used as an index of overall protein breakdown. This approach measures
exclusively long-lived cell proteins which comprise the great majority of protein in most cells (18).

Rates of degradation of these proteins were approximately linear for 18 hr.

Dex caused a consistent 18% increase in the overall rate of protein breakdown (data not shown; \( P<0.05 \)). In the presence of T3, Dex appeared to stimulate protein breakdown by a larger amount (Fig 1a; 22% by 18 hr; \( P<0.01 \)), and this increase was clearly demonstrated within 6 hr. While these changes in overall rates of protein breakdown may appear small, such a 22% increase in the overall degradation rate, if sustained for days, by itself can have an appreciable effect on cell protein content. Furthermore, these seemingly modest changes in overall rates of proteolysis may not reflect much larger increases in degradation of certain components, such as myofibrillar proteins (see below).

Although T3 seems to enhance the effect of glucocorticoids, T3 by itself had no significant effect on overall proteolysis (9% increase ± 2%(SE); \( P>0.10 \)), perhaps because some T3 is already present in the preparation of serum. Nevertheless, to maximize the effect of glucocorticoids, which in vivo are released normally in the presence of T3, we administered dexamethasone together with T3 in most experiments and refer routinely to the combination simply as Dex (unless otherwise noted).

It has been proposed that one component of muscle growth may be an overall reduction in proteolysis (13). To examine whether IGF-1 also affects muscle proteolysis directly in C2C12 cells and/or can counter the increase in protein breakdown induced by glucocorticoids, IGF-1 was added to
the differentiation media alone or 15 min before the addition of Dex (Fig 1a). Within 6 hr after the
addition of IGF-1, overall protein breakdown was reduced by 22% below control levels (P<0.05) and
was 34% lower by 12 hr (P<0.01) than in the Dex-treated cells. Furthermore, the addition of IGF-1
just prior to Dex treatment prevented the increase in proteolysis by Dex (Fig 1a; P<0.01). In other
words, in the presence of Dex plus IGF-1, protein breakdown occurred at rates that did not significantly
differ from control. Thus, the effects of IGF-1 on protein breakdown are dominant over those of
glucocorticoids and fit with the observation in adult organisms that insulin can block the activation of
muscle proteolysis by Dex (54).

Myofibrillar protein breakdown

Myofibrillar proteins are major components of muscle; they are long-lived molecules that are
degraded by the Ub-proteasome pathway (47), and their differential loss is a key feature of muscle
atrophy (9). 3-MH is a post-translationally modified, non-recyclable amino acid which is found in actin
and to a lesser extent in myosin (27). Since 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). Following treatment
with Dex for 30-48 hr, the production of 3-MH by C2C12 cultures increased by 93% (Fig 1b; P<0.05),
which is 3-4 times greater than the increase in overall proteolysis. By contrast, IGF-1 alone appeared
to decrease 3-MH production below basal levels by approximately 27%. Although some reduction was seen consistently, this reduction below control levels did not reach statistical significance ($P>0.10$) because of the large variation in this assay at these low levels of 3-MH. However, when IGF-1 and Dex were added together, 3-MH production was less than half that with Dex alone ($P<0.01$) and again appeared even lower than in control cells by 14%. Thus, IGF-1 and Dex clearly had opposite actions on the breakdown of contractile proteins, and IGF-1 blocked the catabolic effects of Dex. This reduction by IGF-1 is noteworthy since prior studies had concluded that insulin or IGF-1 only affects lysosomal proteolysis and does not reduce the breakdown of myofibrillar proteins (16). While these changes in 3-MH correspond to the Dex-induced changes in overall protein degradation (Fig 1a), the absolute increases (or decreases) in breakdown of myofibrillar components were much larger (93% vs. 22% increase). This differential effect on the degradation of myofibrillar components is likely to be even more important influence on overall proteolysis and muscle protein content in fully differentiated muscle, since in adult muscle two-thirds of cell proteins are components of the myofibril, while the contractile proteins are much less abundant in myotubes.

Effects of IGF-1 and dexamethasone on total RNA and protein content

By definition, muscle atrophy represents a net loss of cell protein and is generally associated with a decrease in total RNA (ribosomal) content, as well as increased proteolysis (Fig 1). To
determine whether these changes in protein breakdown in myotubes are accompanied by loss of RNA, DNA, and/or protein, we measured the effects of treatment with Dex, IGF-1, and Dex plus IGF-1 for 18 hr 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-1 group and the IGF-1 plus Dex group ($P<0.01$). 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, following Dex treatment alone, total protein in the cultured cells decreased by ~10% ($P<0.05$), while if IGF-1 was also present, total protein increased by about 20% ($P<0.01$). Interestingly, the increase in the IGF-1 alone group was smaller and failed to reach significance within 18 hr. (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-1 indicate a stimulation of growth, while 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-1 was present with Dex, there was a clear increase in cell protein and RNA. In other words, the addition of IGF-1 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

In order to determine how Dex or IGF-1 influenced expression of atrophy-related genes, Northern blot analysis and RT-PCR analysis were performed following treatment of myotubes with Dex or IGF-1. By Northern analysis, atrogin-1 mRNA rose significantly within 2 hr following Dex administration (\(P<0.05\); Fig 2). Maximum induction of 2.5-fold was evident by 6 hr and remained at this level through 24 hr of treatment (\(P<0.001\)). However using RT-PCR analysis, a more sensitive method for quantitation of gene expression, we found a 5-fold increase in atrogin-1 expression following Dex treatment (Fig 4a; \(P<0.001\)). Hence, following Dex treatment, atrogin-1 expression appeared to be maximally elevated prior to the increases in protein breakdown and remained elevated when protein breakdown is accelerated, as was found \textit{in vivo} (15).

Poly-Ub mRNA has in the past 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 hr (Fig 2; \(P<0.05\)), but only by 26%, and then remained at this level. Since atrogin-1 mRNA is increased much more than poly-Ub during muscle atrophy \textit{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, both IGF-1 and insulin reduce levels of atrogin-1 mRNA in normal cells
These effects occurred rapidly; for example, IGF-1 or insulin decreased atrogin-1 mRNA by 22% within 30 min and by 70% at 6 hr ($P<0.001$; Fig 3a). In addition, IGF-1 was able to quickly decrease both transcripts of poly-Ub mRNA (2.4 kb and 1.3 kb), although the suppression of the 2.4 kb transcript was twice as great as 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-1 or insulin were added to the media simultaneously, IGF-1 or insulin blocked the increase in atrogin-1 expression by Dex ($P<0.05$; Fig 4a). Furthermore, following pretreatment with Dex for 6 hr to maximally induce atrogin-1, the addition of either IGF-1 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-1 effects are clearly dominant over the ability of glucocorticoids to induce atrogin-1 and to stimulate proteolysis (Fig 1).

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 2-fold increase in MuRF1 expression following Dex treatment ($P<0.05$), which is significantly smaller than the 5-fold increase observed in atrogin-1 mRNA by this approach ($P<0.01$). After addition of IGF-1 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 hr ($P<0.01$), while MuRF1 mRNA only decreased by 36% within 18 hr ($P<0.06$; Fig 4a & c). Furthermore, IGF-1 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% (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 both atrogin-1 and MuRF1 is increased by Dex and suppressed by IGF-1, but atrogin-1 mRNA is much more responsive to these hormonal signals.

**IGF-1 blocks atrogin-1 mRNA synthesis without affecting its degradation**

We tested whether this rapid decrease in atrogin-1 mRNA content by IGF-1 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, with IGF-1, or both. Atrogin-1 mRNA decreased by half within the first hour of actinomycin D treatment and by 74% by 3 hr (Fig 5; $P<0.001$). These effects of actinomycin D closely paralleled the changes in atrogin-1 mRNA seen following IGF-1 treatment, and indicate a short half-life of this mRNA. Moreover, when IGF-1 and actinomycin D were added simultaneously to the cell cultures, the fall in atrogin-1 was similar to the changes seen with IGF-1 treatment alone (Fig 5). Therefore, the effects of IGF-1 on mRNA levels can be explained simply by blocking transcription and evidence on the underlying mechanism will be presented elsewhere (45). Furthermore, IGF-1 and actinomycin D
together did not have a greater effect on atrogin-1 mRNA decay than either alone; so IGF-1 does not appear to promote mRNA breakdown.

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

Expression of other “atrogenes”

Recently, our lab has 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 if other genes induced in atrophy are regulated similarly by glucocorticoids and IGF-1, we measured the levels of several other mRNAs that were highly up-regulated 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 lysosomal cysteine protease, cathepsin L, is the one lysosomal protease that is up-regulated in various types of atrophy (23). As found with atrogin-1, MuRF1, and poly-Ub, mRNA of these atrogenes were increased (as shown by Northern analysis) following Dex
treatment and their expression decreased similarly with IGF-1 addition. Based on these examples, when the atrophy-related transcriptional program is activated by Dex treatment, IGF-1 is able to reverse or block the expression of many atrogens, including metallothionein and cathepsin L (although a more systematic transcriptional array analysis is necessary to test whether all atrogens 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 following treatment with either IGF-1 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) following treatment with either IGF-1 or Dex. This correlation was most sharp when atrogin-1 mRNA content was less than 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-1 and how IGF-1 blocks its induction by Dex. IGF-1 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 either PI3K with LY294002 (LY) or mTOR with rapamycin (Rap) for 6 hr, increased atrogin-1 mRNA levels by approximately 2-fold using both Northern and RT-PCR analysis ($P<0.01$). On the other hand, MuRF1 expression was not significantly elevated following treatment with either inhibitor using Northern analysis and only slightly increased using the more sensitive RT-PCR analysis for Rap treatment only (LY +9.3 ± 6%; Rap +20 ± 5% (SE)). Also, the addition of LY ($P<0.001$), and to a lesser extent Rap ($P<0.01$), for 18 hours (with initial effects observed within 6 hours) increased the rate of breakdown of long-lived proteins in cultured myotubes (Fig 7b).

IGF-1 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 still 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 (U0126 or PD98059) and the calcineurin/NFAT pathway (cyclosporin). Inhibition of these pathways for 18 hr resulted in no change in atrogin-1 mRNA content using 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 being presented 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 hr and decreased 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 nearly 100% as shown by 3-MH production. In myotubes, myofibrillar proteins typically comprise only 1-2% of the total cell protein, while 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-1 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-1 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-1 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-1 when glucocorticoids are in excess could potentially have therapeutic benefits. However, IGF-1 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-α, which has also been proposed to
stimulate muscle atrophy and may reduce the capacity of IGF-1 or insulin to suppress proteolysis (40).

Other pro-inflammatory 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 is important besides the Ub-proteasome pathway, while metallothionein, whose function in atrophy is unknown, curiously showed the largest increase in mRNA following Dex treatment and large increases in vivo during various type of atrophy (23). Poly-Ub mRNA, whose expression has served as the benchmark for activation of Ub-mediated proteolysis in atrophying muscles (55), also increased following Dex administration but to only a 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 (J.M. Sacheck, 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, mRNA's for these E3's are the most sensitive markers for atrophy.

In contrast to Dex, IGF-1 rapidly suppresses both 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-1 suppression of these genes further demonstrate a coordinate regulation of their expression. However, the reduction of atrogin-1 mRNA is especially rapid following IGF-1 administration, while MuRF1 expression falls only slowly. Because the suppression by IGF-1 or insulin of atrogin-1 mRNA is very rapid, this effect is probably a critical early component of their growth-promoting actions. Although IGF-1 given together with Dex prevented MuRF1 induction, IGF-1 treatment following 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 ~1 hr), while MuRF1 mRNA is quite long-lived. Moreover, since the decay of atrogin-1 mRNA after blocking transcription was similar with and without IGF-1, and resembled that when IGF-1 alone was present, the IGF-1 effects appear to be due to its blocking mRNA production, without any effect on mRNA degradation rate. Related studies from this lab demonstrate that IGF-1 and insulin block atrogin-1 expression by inactivating the transcription factors Foxo 1 and 3 which act on the atrogin-1 promoter (45). Moreover, following 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, since 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 atrogin-1 function as an ubiquitin-ligase is tightly linked to protein breakdown and especially to the degradation of myofibrillar proteins. Perhaps atrogin-1 catalyzes directly the breakdown of myofibrillar proteins or influences this process indirectly (e.g. by promoting degradation of proteins that regulate this process); alternately, 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 since 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, both IGF-1 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 of breakdown of contractile proteins, which is a non-lysosomal process (47).

The PI3K/Akt pathway is necessary for muscle hypertrophy (4, 44), and this pathway is activated in response to insulin, IGF-1 and exercise (3, 5). Recently, IGF-1-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-1 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, while inhibition of either PI3K or mTOR increased atrogin-1 expression. In addition, inhibition of PI3K by LY increased the rate of protein breakdown (as did inhibiting mTOR by rapamycin 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-1 and exercise.

Elsewhere we will present evidence that IGF-1, through PI3K and stimulation of Akt phosphorylation and inactivation of the Foxo family of transcription factors, blocks atrogin-1 expression (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-1/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-1, insulin, and exercise -- by reducing degradation of contractile proteins, rapidly suppressing the transcription of atrogin-1 and other key atrogenes, and thus by 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 involve 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 coordinately controlled, and to elucidate how the PI3K/Akt/Foxo pathway is altered in specific conditions associated with muscle wasting.
FOOTNOTES

1The abbreviations used are: Ub, ubiquitin; E3, ubiquitin-ligase; MAFbx, muscle atrophy F-box; MuRF1, muscle ring finger 1; Dex, dexamethasone; IGF-1, insulin-like growth factor-1; PI3K, phosphotidyl-inositol 3-kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; T3, triiodothyronine; 3-MH, 3-methylhistidine; MEK, mitogen-activated protein kinase/extracellular regulated kinase kinase; ERK, extracellular regulated kinase kinase; NFAT, nuclear factor of activated T cells.


3Cathepsin L = GenBank Accession Number - AA174215

4Metallothionein 1 = GenBank Accession Number - NM_013602

5Atrogin-1 - GenBank Accession Number - AW051824

6MuRF1 - GenBank Accession Number - AY059627
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FIGURE LEGENDS

Figure 1. Dexamethasone increases the rate of protein breakdown and myofibrillar protein breakdown, while IGF-1 reduces this process and blocks the stimulation by dexamethasone.

(a) After labeling proteins in C2C12 myotubes for 24 hr with L-[3,5-3H]tyrosine, myotubes were treated with Dex (Dex, 400 ng/ml; T3, 100 ng/ml) and/or IGF-1 (10 ng/ml) and release of L-[3,5-3H]tyrosine from cell proteins was used as an index of the breakdown of long-lived proteins. Panel 1 illustrates the rate of protein breakdown calculated by the change in percentage of total incorporated L-[3,5-3H]tyrosine over 18 hr relative to control. △, ▲, ○, and ■ represent control, Dex, IGF-1, and Dex + IGF-1 group, respectively. Panel 2 displays the differences in the rates of protein breakdown at 18 hr when the effects of these treatments are maximal. (b) Myofibrillar protein breakdown, measured by 3-methylhistidine release, following treatment with Dex (Dex, 400 ng/ml; T3, 100 ng/ml) and/or IGF-1 (10 ng/ml) for 30-48 hr. Values are means ± SD, (n=5-6/group). *(P<0.05) vs. control, *(P<0.01) vs. Dex.

Figure 2. Treatment of myotubes with dexamethasone rapidly increases atrogin-1 and poly-ubiquitin mRNA. At the outset, myotubes (n=4/group) were treated with Dex (400 ng/ml), followed by Northern analysis (10 µg of total RNA loaded per lane), and probed with atrogin-1, poly-Ub,
and GAPDH.

**Figure 3.** IGF-1 rapidly suppresses atrogin-1 and poly-ubiquitin mRNA in C2C12 myotubes. (a) Myotubes were treated with IGF-1 (10 ng/ml) for 3 hr. cDNA levels of mouse atrogin-1 and GAPDH were assessed by real-time PCR. IGF-1 decreased atrogin-1 mRNA within 30 minutes ($P<0.05$) and by 70% within 3 hours ($P<0.001$). (b) Total RNA from control and IGF-1 treated myotubes were probed and quantitated by Northern analysis (10 µg of total RNA loaded per lane) for poly-Ub mRNA. Graph: 2.4 kb transcript = poly-Ub A, 1.3 kb transcript=poly-Ub B, average of both transcripts = poly-Ub A&B. Values are expressed relative to control (n=4/group) and results were reproduced in at least three independent experiments.

**Figure 4.** IGF-1 and insulin block dexamethasone-induced atrogin-1 and MuRF1 expression in C2C12 myotubes. (a) Real-time PCR analysis of atrogin-1 and MuRF1 expression following 6 hr treatment with Dex (Dex, 400 ng/ml; T3, 100 ng/ml), and/or IGF-1 (10 ng/ml) or insulin (1 µg/ml). (b) Real-time PCR analysis of atrogin-1 expression following 6 hr treatment with Dex followed by the addition of IGF-1 (10 ng/ml) or insulin (1 µg/ml) for an additional 6 hr. Addition of IGF-1 or insulin to the myotubes following 6 hr treatment with Dex did not reverse the Dex-induced increase in MuRF1
expression (data not shown). (c) Real-time PCR analysis demonstrating the rapidity and magnitude of suppression of atrogin-1 and MuRF1 mRNA in C2C12 myotubes following treatment with IGF-1 (10 ng/ml). Values are means ± SE (n=4-5/group). *at least (P< 0.05) vs. control, +at least (P<0.01) vs. Dex, ++(P<0.001) vs. Dex.

Figure 5. IGF-1 rapidly reduces atrogin-1 mRNA by blocking its transcription without affecting mRNA degradation. Real-time PCR analysis of the effect of actinomycin D (5 µg/ml), IGF-1 (10 ng/ml), or the combination of actinomycin D plus IGF-1 treatment on the expression of atrogin-1 and MuRF1 mRNA. Values are expressed relative to control (n=4/group) and results were reproduced in at least three independent experiments.

Figure 6. Overall protein breakdown in myotubes correlates highly with atrogin-1 mRNA but not with MuRF1 mRNA. Values of atrogin-1 mRNA and protein breakdown from identical myotube cultures following 18 hr treatment with IGF-1 (10 ng/ml) or Dex (Dex, 400 ng/ml; T3, 100 ng/ml) and expressed as a percentage of control. A highly significant linear relationship was found between atrogin-1 expression (up to 200% of control) and protein breakdown values (70-110% of control) (R²=0.79, P<0.001), while values above these demonstrated a different linear relationship (R²=0.49,
Measurement of MuRF1 mRNA and protein breakdown did not result in a similar correlation (data not shown; R²=0.14; P>0.10)

Figure 7. Inhibition of the PI3K/Akt/mTOR pathway (but not the calcineurin/NFAT or MEK1/2 pathways) increases atrogin-1 expression and protein breakdown.  (a) Northern blot showing the effect of 6 hr treatment with LY294002 (LY; 10 μM), rapamycin (Rap; 2 ng/ml), or IGF-1 (10 ng/ml) on atrogin-1 expression in C2C12 myotubes (10 μg of total RNA loaded per lane). There are two major transcripts (~2.4 and 7.5 kb) of atrogin-1 in myotubes.  (b) Cell proteins of C2C12 myotubes were pre-labeled and L-[3,5-3H]tyrosine release was used as an index of overall protein breakdown (as in Fig1).  At the outset, myotubes were treated with LY (10 μM), Rap (2 ng/ml) or IGF-1 (10 ng/ml).

The rate of protein breakdown is expressed as the change in the % of protein degraded/hour relative to the amount of L-[3,5-3H]tyrosine incorporated over 18 hr relative to control.  (c) Real-time PCR analysis illustrating atrogin-1 expression in C2C12 myotubes treated with LY (10 μM), Rap (2 ng/ml), cyclosporin (CsA, 1 μM), U0126 (50 μM), or PD98059 (10 μM) for 18 hr.  Values are means ± SE (n=4-5/group).

*at least (P< 0.05) vs. control, *(P<0.001) vs. IGF-1.
Table I. Dexamethasone treatment decreases total myotube content of RNA and protein while IGF-1 promotes these processes and blocks the effects of dexamethasone.

<table>
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<tr>
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<th>Control</th>
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<th>IGF-1</th>
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<tr>
<td>Total RNA</td>
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<td>108 ± 22%</td>
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Values were taken at 18 hours post-treatment, were quantitated as mg/plate, and are means ± SD (n=4). * (P< 0.05), § (P<0.01) vs. control, at least † (P<0.01) vs. Dex.
Table II. Dexamethasone treatment increases the expression of atrophy-related genes ("atrogenes") in C2C12 myotubes, while IGF-1 suppresses their expression and blocks the effects of dexamethasone.

<table>
<thead>
<tr>
<th>Atrogene</th>
<th>Dex (% control)</th>
<th>IGF-1 (% control)</th>
<th>Dex + IGF-1 (% control)</th>
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<tr>
<td>Atrogin-1</td>
<td>530%**</td>
<td>30%**§§</td>
<td>35%§§</td>
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<td>MuRF1</td>
<td>210%**</td>
<td>64%§§</td>
<td>101%§</td>
</tr>
<tr>
<td>Poly-Ub</td>
<td>143%**</td>
<td>58%**§</td>
<td>117%§†</td>
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<tr>
<td>Metallothionein</td>
<td>463%**</td>
<td>74%§§</td>
<td>112%§§</td>
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<tr>
<td>Cathepsin L</td>
<td>172%*</td>
<td>83%§§</td>
<td>108%§</td>
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</table>

Results are quantitated by Northern analyses (except atrogin-1 and MuRF1 – analyzed by RT-PCR) as described in methods and are average values from 2-6 experiments per gene. Values are expressed as % of untreated control. *(P< 0.05) and at least **(P<0.01) vs. control, §(P<0.05) and at least §§(P<0.01) vs. Dex-treated, and †(P<0.05) vs. IGF-1 treated.
Figure 1

(a) Time course of L-[3,5-3H]tyrosine release (% of total radioactivity) in response to different treatments: Control, IGF, DEX, and DEX+IGF.

(b) Rate of protein breakdown (% of control) for different treatments: Control, Dex, IGF-1, and Dex + IGF.

(c) 3-Methylhistidine release (% of control) for different treatments: Control, Dex, IGF-1, and Dex + IGF.
**Figure 2**

<table>
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</table>
Figure 4

(a) Bar graph showing fold change in Atrogin-1 and MuRF1 expression under different conditions: Control, Dex, IGF-1, Insulin, and Dex+IGF. The bars for Atrogin-1 are labeled with symbols indicating significant differences.

(b) Similar bar graph as in (a) with a note of "6 hr pre-treatment with Dex." The bars for Atrogin-1 are labeled with symbols indicating significant differences.

(c) Line graph showing the percentage of Control for Atrogin-1 and MuRF1 over time (hours following IGF-1 treatment).
Figure 5

![Atrogin-1 and MuRF1 graphs](image-url)
Figure 6

Protein Breakdown (% of control) vs. Atrogin-1 mRNA (% of control)

- Dex: $R^2 = 0.49$
- IGF-1: $R^2 = 0.79$
Figure 7

(a) Western blot analysis of Atrogin-1 expression.

(b) Bar graph showing the rate of protein breakdown (% of control) for different conditions: Control, IGF-1, LY, and Rap. The bars indicate the fold change relative to the control, with statistical significance indicated by + or *.

(c) Bar graph depicting the fold change of Atrogin-1 expression for various treatments: Control, LY, Rap, CsA, U0126, and PD98059. Statistical significance is marked by *.