Hindlimb casting decreases muscle mass in part by proteasome-dependent proteolysis but independent of protein synthesis


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Krawiec, Brian J., Robert A. Frost, Thomas C. Vary, Leonard S. Jefferson, and Charles H. Lang. Hindlimb casting decreases muscle mass in part by proteasome-dependent proteolysis but independent of protein synthesis. Am J Physiol Endocrinol Metab 289: E969–E980, 2005. First published July 26, 2005; doi:10.1152/ajpendo.00126.2005—The hypothesis of the present study was that rats subjected to short-term unilateral hindlimb immobilization would incur skeletal muscle wasting and concomitant alterations in protein synthesis, controllers of translation, and indexes of protein degradation. Rats were unilaterally casted for 1, 3, or 5 days to avoid complications associated with other disuse models. In the casted limb, gastrocnemius wet weight decreased 12% after 3 days and thereafter remained constant. In contrast, the contralateral control leg displayed a steady growth rate over time. The rate of protein synthesis and translational efficiency were unchanged in the immobilized muscle at day 5. The total amount and phosphorylation state of regulators of translational initiation and elongation were unaltered. The mRNA contents of polyubiquitin and the ubiquitin ligases muscle atrophy F-box (MAFBx)/Atrogin-1 and muscle RING finger 1 (MuRF1) were elevated in immobilized muscle at all time points, with peak expression occurring at day 3. Daily injection of the type II glucocorticoid receptor antagonist RU-486 did not prevent decreases in gastrocnemius wet weight nor increases in mRNA for MAFBx/Atrogin-1 and MuRF1. However, in vivo administration of the proteasome inhibitor Velcade prevented 53% of wet weight loss associated with 3 days of immobilization. These data suggest that the loss of skeletal muscle mass in this model of disuse appears to be glucocorticoid independent, can be partially rescued with a potent proteasome inhibitor, and is associated with enhanced mRNA expression of multiple factors that contribute to ubiquitin-proteasome-dependent degradation and are likely to control the remodeling of immobilized skeletal muscle during atrophy.

Atrogin-1; muscle RING finger 1; disuse; eukaryotic initiation factor; translation

Skeletal muscle demonstrates a remarkable degree of plasticity in response to alterations in mechanical loading. Removal of normal weight-bearing stress results in quantitative and qualitative adaptations in protein content that are phenotypically epitomised by decreased muscle wet weight, fiber diameter, force output, fatigue resistance, and a slow-to-fast transition of myosin isoform expression. Changes in tissue mass are primarily determined at a cellular level by complex signaling pathways (19) that coordinate growth and proliferation (11). These circuits ultimately manage specific enzymatic machinery with intimate ties to fundamental biological events such as macromolecular synthesis or degradation and gene expression. Indeed, the etiology of atrophy in various models of mechanical unloading or “disuse” is attributable to both a repression in protein synthesis and/or an elevation in protein degradation (6, 8, 20, 21, 45, 48, 49).

Control of protein synthesis is predominantly exerted during the process of mRNA translation (27). In this regard, protein synthesis can be regulated by two mechanisms: alteration of the ribosomal content (the base translational capacity of a cell) or of the translational activity of preexisting ribosomes (the efficiency or rapidity with which they participate in cyclical rounds of translation). Acute repression of protein synthesis with casting immobilization is not a consequence of significant defects in total RNA concentration (6, 20), but instead can be attributed to a reduction in translational efficiency (20). Control of the translational rate is primarily exerted at two main loci, initiation and elongation, and is exercised in part by a cadre of eukaryotic initiation (eIF) and elongation (eEF) factors (27). Many of these are phosphoproteins whose function is dependent on inputs from various intracellular kinases.

The connection between the control of organism, organ, and cell mass and a wide variety of extracellular cues has led to the characterization of several canonical, evolutionarily conserved growth pathways, the phosphatidylinositol 3-kinase (PI 3-kinase)/protein kinase B (Akt)/glycogen synthase kinase-3 (GSK3) and mammalian target of rapamycin (mTOR)/S6K/4E-binding protein (BP)-1 pathways (19). These pathways serve critical functions in controlling adaptive responses of skeletal muscle in part by matching the mobilization of the translational machinery (i.e., eIFs, eEFs, and ribosomal subunits) with the requirement for changes in cellular mass. Akt is activated proximally by the concerted action of PI 3-kinase and 3-phosphoinositide-dependent protein kinase 1 (PDK-1), and consequently signals directly or indirectly to a number of important controllers of protein synthesis, including GSK3 (5, 41, 51) and mTOR and its effectors S6K1 and 4E-BP1 (5, 40–42). Importantly, hindlimb suspension-induced atrophy of antigravity muscles is associated with decreased protein expression and phosphorylation of Akt (5). These last observations are indicative of a direct responsiveness of Akt to the loading state of muscle that correlates with the allied muscular atrophy.

mTOR, in association with various adaptor proteins (23), forms two distinct multimeric complexes that function in control of cell size and number through a variety of processes, including ribosomal biogenesis, cap-dependent translation, and cytoskeletal organization. The best-characterized downstream targets of mTOR are S6K1 and 4E-BP1. S6K1 plays obligatory roles in promoting organism growth (43), presumably by regulating both the general rate of translation through stimulation of eIF4A and eIF4B (39) and specific translation of...
5’-terminal oligopyrimidine (5’-TOP) mRNA (26). 4E-BP1 is the isoform of the translational repressor 4E-BP family predominantly expressed in skeletal muscle and is a negative regulator of cell growth (15). Similarly to Akt, a reduction of skeletal muscle loading with hindlimb suspension decreases the activation state of S6K1 (5) and increases the amount of 4E-BP1 bound to eIF4E (5), suggesting the pathology of disuse muscle wasting can be traced to a signaling defect in the mTOR/S6K1/4E-BP1 pathway.

Of four distinct proteolytic activities expressed in skeletal muscle, the ATP-dependent ubiquitin-proteasome system appears to predominate under conditions of wasting or atrophy induced by a variety of conditions (35), including hindlimb suspension (45). There are two functional facets to this system: the targeting of specific proteins for degradation and their ultimate destruction by the proteasome. The targeting requirement is fulfilled by the activity of a hierarchical cascade of E1, E2, and E3 enzymes in concert with the labeling cofactor ubiquitin. Because ubiquitin conjugation is thought to be the rate-limiting step of degradation, the upregulation of specific E2s and E3s and ubiquitin may be central to the atrophic response during catabolic states. This notion is supported by gene expression studies demonstrating that disparate atrophic stimuli drive the induction of a specific transcriptional program, resulting in increased expression of components innate to the ubiquitin-proteasome system (31, 44). In particular, the muscle-specific E3 ligases muscle atrophy F-box (MAFbx)/Atrogin-1 (4, 22) and muscle RING finger 1 (MuRF1; see Ref. 4) are increased by a multitude of catabolic perturbations, such as treatment with IL-1 (4) or dexamethasone (4) as well as cancer cachexia (22, 31), diabetes (22, 31), fasting (31), and sepsis (53). Together, these data indicate that activation of a specific transcriptional response to catabolic cues activates the ubiquitin-proteasome system and consequently enhances proteolysis.

Collectively, the aforementioned studies establish a role for several critical events and signaling pathways in control of skeletal muscle cell size under a variety of conditions of normal use and disuse. However, this body of knowledge represents an accrual of results obtained using a wide breadth of atrophic models, time courses, and examined muscles. Therefore, no complete set of information exists for one well-defined model of disuse atrophy. Hence, the purpose of these studies was to examine protein synthesis and translational control pathways in a clinically and physiologically relevant model of disuse atrophy and transcriptional upregulation of specific atrophic pathways associated with protein synthesis, separate groups of rats were injected intravenously with either IGF-I (25 nmol/kg body wt), insulin (5 U/kg body wt), or an equivalent volume (0.5 ml) of isotonic saline after thermal injury (29). In a separate experiment, IGF-I was injected subcutaneously once daily with the polyethylene glycol 4000–conjugated 19-nortestosterone (250 mg/kg body wt; Sigma-Aldrich, St. Louis, MO) or an equivalent volume (0.5 ml) of vehicle (60% ethanol-40% saline) for the duration of the experiment. This dose of RU-486 has been used previously by this laboratory to attenuate glucocorticoid-induced changes in protein and mRNA content after thermal injury (29). In a separate experiment, rats were injected intraperitoneally at the time of casting with Velcade (0.5 mg/kg body wt; Millennium Pharmaceuticals, Cambridge, MA) or an equivalent volume (0.5 ml) of vehicle (saline). This dose has been used previously to study proteasome function in vivo (9, 10) and attenuates muscle wasting associated with the use of glucocorticoids in vivo (11). In a separate experiment, rats were injected intraperitoneally at the time of casting with Velcade (0.5 mg/kg body wt; Millennium Pharmaceuticals, Cambridge, MA) or an equivalent volume (0.5 ml) of vehicle (saline). This dose has been used previously to study proteasome function in vivo (9, 10).

Materials and Methods

Animals. Pathogen-free male Sprague-Dawley rats (150–175 g; Charles River Breeding Laboratories, Cambridge, MA) were quarantined for 1 wk while exposed to constant temperature and a 12:12-h light-dark cycle. Standard rat chow (Harlan Teklad, Indianapolis, IN) and water were provided ad libitum. All experiments were performed in adherence with the National Institutes of Health Guide for Care and Use of Laboratory Animals and with the approval of The Pennsylvania State University College of Medicine Institutional Animal Care and Use Committee.

Experimental protocols. Rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (50 mg/kg) and subjected to unilateral hindlimb immobilization via a fiberglass cast. Briefly, the left hindlimb was shaved and wrapped in a protective layer of cast padding (Specialist brand; Johnson and Johnson, Raynham, MA). Multiple layers of fiberglass casting tape (3M VetCast Plus veterinary casting tape; 3M, St. Paul, MN) were then applied and allowed to harden. The foot was positioned in plantar-flexion to induce maximal atrophy of the gastrocnemius (20, 21, 54). After being cast, rats were resuscitated with 10 ml of 0.9% sterile saline administered subcutaneously. Results from pilot studies (data not shown) indicated that unilateral immobilization had no effect on various parameters of interest in skeletal muscle from the contralateral noncasted leg. Consequently, the contralateral hindlimb served as the control in all subsequent experiments. After casting, rats were housed individually and provided free access to standard rat chow and water.

Immobilization was imposed for a duration of 1, 3, or 5 days. After an overnight fast, casts were removed under pentobarbital sodium anesthesia, and gastrocnemius from both the immobilized and control limbs was harvested. Results from pilot studies (data not shown) indicated that unilateral immobilization had no effect on parameters of interest in skeletal muscle from the contralateral noncasted leg. Consequently, the contralateral hindlimb served as the control in all subsequent experiments. After casting, rats were housed individually and provided free access to standard rat chow and water.

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alcoholic hepatitis models (3, 7, 30). After 3 days of immobilization, muscles were processed as above.

**Protein synthesis, RNA content, and translational efficiency.** The rate of muscle protein synthesis was determined in vivo by the flooding-dose technique (12, 17, 28). Casts were removed, and a catheter was placed in the carotid artery. At time 0, rats were administered either IGF-I or an equal volume of isotonic saline via percutaneous injection of the inferior vena cava. At the 10-min time point, all rats received a bolus injection of 1-(^3)Hphenylalanine (150 mM, 30 μCi/ml, 1 ml/100 g body wt) via the jugular vein. Blood samples (for determination of phenylalanine concentrations and specific radioactivity) were collected at 12, 16, and 20 min via the arterial catheter in heparinized syringes. Gastrocnemius was then excised and frozen as above. Total protein concentration of immobilized and control gastrocnemius was determined on powdered tissue via the Biuret method with a crystalline BSA standard. A fraction of powdered gastrocnemius was weighed, dried for 2 days in an oven (70°C), and reweighed to calculate the dry weight-to-wet weight ratio.

The specific radioactivity of deproteinized plasma was determined by HPLC (13). Specific radioactivity was calculated by dividing the radioactivity of the phenylalanine peak by the concentration of phenylalanine in the sample. A portion of powdered muscle was used to determine the rate of incorporation of radioactive phenylalanine in protein, as previously described (12, 28). Total RNA was determined by spectrophotometry using previously described methods (12, 28). Translational efficiency was calculated by dividing the rate of protein synthesis of mixed-muscle proteins by the total RNA concentration and expressed as nanomoles of phenylalanine incorporated into mixed protein per hour per milligram of RNA. The vast majority of cellular RNA in skeletal muscle is ribosomal; therefore, measurements of total tissue RNA provide an accurate reflection of ribosomal content and are appropriate for the calculation of efficiency.

**Immunoblotting and immunoprecipitations.** The protein concentration of tissue supernatants was determined by Bradford assay (Bio-Rad Laboratories, Hercules, CA). Sample buffer (2×) was added to an equal volume of tissue supernatant. Samples were loaded according to total protein content on polyacrylamide gels for separation by SDS-PAGE. Proteins were transferred to a polyvinylidene difluoride membrane (Biotrace; PALL, Pensacola, FL), blocked in nonfat dry milk, and incubated overnight at 4°C with radiolabeled antibodies for the following proteins: Akt (Thr^308), mTOR (Ser^2448 and Ser^2481), S6K1 (Thr^4290), ribosomal protein S6 (rpS6; Ser^235/236 and Ser^240/244), 4E-BP1 (Thr^766), eIF4G (Ser^1108), eIF2α (Ser^51), eIF2 (Thr^56), tuberin (Thr^1462) (all from Cell Signaling Technology, Beverly, MA), and eIF2β (Ser^58; Biosource International, Camarillo, CA). Excess primary antibody was removed by washing in 1× Tris-buffered saline + 0.1% Tween 20, and membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse secondary antibody (Sigma-Aldrich) at room temperature. Blots were developed using enhanced chemiluminescence (Amersham Biosciences; a rat 18S oligonucleotide-5′-GGATATTACAGC-3′, MAbfx/Atrogin-1-5′-CCCACCAAGCGGACTTGCGACTCTTGACCGCTG-3′, MuRF1-5′-AGCGGAACGACTCCAGACATGGAGCAGGACCCGGC-3′), and polyubiquitin (5′-GGATTTTGCCCCTACGTTCTGAGTGGTGTTTGACCTGGCCTC-3′) labeled by terminal transferase (Roche Diagnostics, Indianapolis, IN) tailing with [α-32P]dATP (Amersham Biosciences); a rat 18S oligonucleotide-5′-GGATATTACAGC-3′ was identically labeled. A rat IGF-I probe was generated from cDNA (provided by Peter Rotwein, St. Louis, MO), using a Random Primed DNA Labeling kit (Roche). Membranes were washed two times in 2× saline-sodium citrate (SSC)-0.1% SDS at 42°C for 5 min, one time in 0.2× SSC-0.1% SDS at 42°C for 15 min, and one time in 0.2× SSC-0.1% SDS at 48°C for 10 min to remove unbound probe. Blots were exposed to a PhosphorImager screen (Molecular Dynamics, Sunnyvale, CA), visualized, and analyzed using ImageQuant software (version 5.2; Molecular Dynamics). Signal densities for mRNAs were normalized to densities for 18S mRNA. Cytokine mRNA was determined by RiboQuant Multi-probe RNase Protection Assay (BD PharMingen, San Diego, CA). Riboprobes were generated with the rat tRNA-1 template set by use of an in vitro transcription kit (BD PharMingen). Riboprobes were hybridized with 20 μg of total RNA as per the manufacturer’s protocol, and protected RNAs were detected by electrophoresis on vertical 5% acrylamide gels. Gels were dried and exposed to PhosphorImager screens as outlined above. Data were analyzed using ImageQuant software and normalized to L32 mRNA.

**Statistics.** Experimental values are presented as means ± SE. Data were analyzed by pairwise Student’s t-test in two-group comparisons and ANOVA followed by Student-Neuman-Keuls test in multigroup comparisons to determine treatment effect when ANOVA indicated a difference among the means. Differences between groups were considered significant at P < 0.05.

**RESULTS**

Unilateral immobilization induces skeletal muscle loss. Collectively, data from several separate studies indicated that 5 days of hindlimb immobilization decreased muscle wet weight 20–30% relative to time-matched contralateral control muscle. From the time-course data presented in Fig. 1, two distinct
Fig. 1. Effect of 1, 3, and 5 days of immobilization on gastrocnemius wet weight. The value from the contralateral control gastrocnemius of rats subject to 1 day immobilization is set at 100%. Data are presented as a line graph of the mean percentage of gastrocnemius weight compared with the 1-day control ± SE for 10 animals/group. Means with different letters are statistically different from one another (*P < 0.05). Average immobilized and contralateral control gastrocnemius wet weights (in grams) were 1.01 and 1.05 after 1 day, 0.92 and 1.12 after 3 days, and 0.93 and 1.32 after 5 days, respectively.

Processes appear to underlie this wasted phenotype. No significant change was seen in wet weight after a single day of immobilization. By the 3rd day, the gastrocnemius of the casted limb was significantly smaller than both the time-matched contralateral muscle (~19% at day 3; Fig. 1, point b vs. c) and the 1-day immobilized gastrocnemius (~8%; Fig. 1, point b at day 3 vs. point a at day 1); this latter observation is demonstrative of skeletal muscle atrophy. By the 5th day, the weight of the casted muscle was considerably reduced compared with control values (Fig. 1, point b at day 5 vs. point d). However, unlike immobilized wet weight comparisons between days 3 and 1, there was no difference in the size of the casted gastrocnemius between days 5 and 3 (Fig. 1, point b at day 5 vs. point b at day 3). This latter response is more indicative of growth failure in the muscle of the casted limb relative to the contralateral weight-bearing limb.

There was a concordant decrease in both total muscle protein concentration (179.4 ± 4.7 vs. 160.1 ± 3.9 mg protein/g wet wt, control vs. immobilized muscle, *P < 0.05) and protein content (306.7 ± 10.1 vs. 215.9 ± 6.5 mg protein/whole muscle, control vs. immobilized muscle, *P < 0.05) after 5 days of immobilization. Additionally, the calculated dry weight-to-wet weight ratios indicated a small but statistically significant decrease of the casted gastrocnemius (23.2 ± 0.2 vs. 22.6 ± 0.2%, control vs. immobilized muscle, *P < 0.05).

Activation of Akt. Full activity of Akt in response to stimulation by growth factors is dependent on phosphorylation at two residues, Thr308 and Ser473. Casted and control gastrocnemius from animals immobilized for 5 days displayed a comparable basal Akt phosphorylation that was enhanced equivalently in both muscles severalfold by IGF-I (Fig. 2). In the casted gastrocnemius, total Akt protein decreased in proportion to the loss in protein content. However, the phosphorylation noted above was not a consequence of an alteration in the relative expression of total Akt when equivalent amounts of protein from immobilized and control samples were subject to SDS-PAGE. Hereafter, this relative expression per equal amount of muscle protein will be referred to as the “total” amount.

Additionally, Akt plays an established role in PI 3-kinase-dependent phosphorylation of tuberin, the product of the tumor-suppressor gene TSC2. Tuberin is a negative regulator of cell growth via its formation of a heterodimer with hamartin (the product of the TSC1 gene), consequently creating a functional complex capable of depressing activity of mTOR and its related effectors (24). These growth-suppressing properties can be precluded by Akt-directed phosphorylation of tuberin on Thr1462 (33). Five days of immobilization was without effect on tuberin phosphorylation or content in any context (data not shown), arguing against repression of the TSC/Rheb/mTOR signaling pathway under these experimental conditions.

Phosphorylation of S6K1 and rpS6. The laddering effect of S6K1 seen with IGF-I (Fig. 3A) is indicative of decreased electrophoretic mobility of the protein because of conformational changes in structure via phosphorylation. Thr389 in the hydrophobic motif of the linker region is the principal rapamycin-sensitive, and therefore mTOR activity-dependent, residue whose phosphorylation is requisite for full kinase activity (38). Basal Thr389 phosphorylation was virtually undetectable in
immobilized and control muscle from saline-treated rats (Fig. 3B). IGF-I administration resulted in a marked multifold increase in phosphorylation, the magnitude of which was not different between casted and control muscle (Fig. 3B). Moreover, this phosphorylation was not a result of an increased protein expression, since total S6K1 remained unchanged under all conditions.

rpS6 is a component of the 40S ribosome and is postulated to play a role in mediating the effects of S6K1 on 48-TOP translation. Furthermore, under many conditions, phosphorylation of rpS6 correlates with increases in translational initiation and elongation rates (10, 14). Constitutive phosphorylation at two sets of sites in rpS6 (Ser235/236 and Ser240/244) was increased in 5-day-immobilized gastrocnemius (Fig. 4A and B). This immobilized-induced increase in basal phosphorylation was greater for Se1108/h/1236/236 (~3-fold) relative to Se1108/h/1230/244 (~50%). IGF-I treatment caused a disparate site-dependent increase in phosphorylation of rpS6. Growth factor-stimulated phosphorylation of Ser235/236 was equivalent between immobilized and control groups, whereas phosphorylation of Ser240/244 was induced to a greater extent in muscle from the casted limb (Fig. 4B). These changes occurred in the absence of altered total amounts of rpS6.

Phosphorylation of 4E-BP1, eIF4G, and mTOR and the distribution of eIF4E. When resolved by SDS-PAGE, 4E-BP1 separates into three distinct bands (γ, β, and α, in order of hyper- to hypophosphorylation). Analysis of the γ-isoform of 4E-BP1 was performed with a site-specific antibody for phosphorylation at Thr37/46, an mTOR-dependent process thought to serve as a priming event for additional subsequent phosphorylation events that result in release of bound eIF4E (18). After 5 days of immobilization, there was no difference in the γ-form between casted and control muscle under basal conditions (Fig. 5A). Additionally, both muscles displayed similar increases in phosphorylation after growth factor treatment (Fig. 5A).

The proportion of eIF4E associated with 4E-BP1 (Fig. 5B) and eIF4G (Fig. 5C) was not different in gastrocnemius from casted and control limbs under basal conditions. Treatment with IGF-I caused a dissociation of eIF4E with 4E-BP1 and a concomitant increase in association of eIF4E with eIF4G (Fig. 5, B and C, respectively) that was comparable in both groups and correlated with the above-mentioned growth factor-induced phosphorylation of 4E-BP1 (Fig. 5A).

There was no difference in the extent of basal Ser1108 phosphorylation of eIF4G between immobilized and control muscle after 5 days of immobilization (Fig. 5D). Both muscles remained responsive to the actions of IGF-I, although the increment in the muscle from the casted limb was significantly lower than that seen in control muscle. These observations were independent of a change in the total amount of eIF4G.

Finally, the phosphorylation of mTOR at Ser2448 and Ser2481 was not significantly different in saline-treated immobilized and control muscle under basal conditions. Furthermore, stimulation with IGF-I resulted in equivalent increases in the phosphorylation at both residues, regardless of muscle load (data not shown).

Subunit-specific changes in guanine nucleotide-exchange factor eIF2B. Activation of eIF2 and formation of the 43S preinitiation complex is facilitated by eIF2B, a multisubunit enzyme that expresses GDP-GTP nucleotide exchange factor eIF2B. The largest subunit, α, serves as a substrate for several protein kinases, and its activity can be modulated by these phosphorylation events (50). There were no significant differences in eIF2Be phosphorylation at Ser535 between any treatment after 5 days of hindlimb immobilization (data not shown). The five subunits of eIF2B associate to form two distinct subcomplexes whereby the ε- and γ-subunits form a catalytic subcomplex and the α-, β-, and δ-subunits form a regulatory subcomplex (37). Immobilization induced a subcomplex-dependent increase in total subunit expression. The casted muscle exhibited an increase in total eIF2Be protein compared with control plus saline values (1,153 ± 224 vs. 1,829 ± 180 arbitrary units, control vs. immobilized muscle, P < 0.05). This same augmentation was not observed for the β-subunit, where no change in total protein was seen under any condition (data not shown). An additional mechanism of translational initiation control involves the phosphorylation of the
α-subunit of eIF2 at Ser51, which effectively alters its role from substrate to a competitive inhibitor for the guanine nucleotide exchange factor activity of eIF2B. No change in phosphorylation or total amount of eIF2α was noted with immobilization or IGF-I treatment (data not shown).

Protein synthesis and translational efficiency. When mixed-muscle protein synthesis was assessed directly, no difference was observed between any of the experimental conditions (Table 1). Although the total RNA content of the casted gastrocnemius decreased, when normalized to muscle wet weight, the total RNA concentration was found to be equivalent to that of control muscle (Table 1). Translational efficiency was not reduced significantly in immobilized muscle (Table 1). No change in any of the above parameters was detected after 20 min of IGF-I stimulation.

Effect of insulin on regulators of translational initiation. Because the observed decrement in wet weight with casting could be a result of resistance to humoral anabolic cues, the acute effects of insulin on regulatory proteins implicated in 5’-TOP and cap-dependent translation were also investigated after 5 days of immobilization. Casted and control gastrocnemius muscle remained equally responsive to the stimulatory effects of insulin and displayed qualitatively similar results to those obtained after IGF-I administration regarding phosphorylation of Akt, S6K1, rpS6, 4E-BP1, eIF4G, and mTOR (data not shown).

Gene expression of MAFbx/Atrogin-1, MuRF1, and polyubiquitin. Immobilization resulted in a rapid and persistent increase in both MAFbx/Atrogin-1 (Fig. 6A) and MuRF1 (Fig. 6B) mRNA in the casted muscle compared with control. For both genes, expression was significantly upregulated at day 1, attained a peak at day 3, and returned to day 1 values at day 5. It is noteworthy that the maximum expression of these genes is associated with immobilization-induced atrophy (compare day 3, Figs. 1, 6A, and 6B), whereas growth failure coincides with the lower level of induction (compare day 5, Figs. 1, 6A, and 6B).

The time course of induction for the polyubiquitin gene family (31) in response to unilateral unloading was identical to that determined for the ubiquitin ligases (Fig. 7A). This stimulation of the polyubiquitin gene concurs with a qualitative assessment of increased ubiquitin conjugates in immobilized and control muscle over 1, 3, and 5 days (Fig. 7B). Ubiquitin antibody immunoreactivity is increased in immobilized muscle homogenates (compare immobilized vs. control conditions) and was greatest at day 3, allied with peak mRNA expression.

Gene expression of IGF-I, myostatin, and proinflammatory cytokines. Muscle loss during immobilization may also be a consequence of altered expression of other positive or negative eEFs. The elongation stage of eukaryotic translation is dependent on the function of two distinct factors, eEF1A and eEF2 (27). No change in protein content was seen for either factor, nor was the phosphorylation state of eEF2 altered in response to immobilization or IGF-I treatment (data not shown).

Fig. 4. Effect of 5 days of immobilization on phosphorylation of ribosomal protein S6 (rpS6). A, top: densitometric analysis of rpS6 phosphorylation at Ser235 and Ser236. The value from contralateral control gastrocnemius of rats given saline (iv) is set at 1.0 AU. A, bottom: representative immunoblots of phosphorylation at the Ser235 and Ser236 sites of rpS6 and total rpS6, respectively. B, top: densitometric analysis of rpS6 phosphorylation at Ser240 and Ser244. The value from contralateral control gastrocnemius of rats given saline (iv) is set at 1.0 AU. Data are presented as a bar graph of the mean ± SE for 12 animals/group. Means with different letters are statistically different from one another (P < 0.05). B, bottom: representative immunoblots of phosphorylation at the Ser240 and Ser244 sites of rpS6 and total rpS6, respectively.
regulators of growth and tissue mass. In this regard, 5 days of immobilization did not result in a statistically significant change in IGF-I, myostatin, tumor necrosis factor-α, IL-6, or IL-1β mRNA between casted and control muscle (data not shown).

Immobilization-induced atrophy is glucocorticoid independent. To determine if an altered sensitivity of casted muscle to glucocorticoids was a causal factor in the observed wasting and gene expression for the E3 ligases MAFbx/Atrogin-1 and MuRF1, rats were subjected to unilateral hindlimb immobilization for 3 days while concomitantly treated with vehicle or the glucocorticoid receptor antagonist RU-486. There was no significant difference in skeletal muscle wet weight or mRNA expression of MAFbx/Atrogin-1 and MuRF1 between vehicle and RU-486-treated immobilized muscle, as was the case for vehicle and antagonist-treated control muscle (data not shown).

Immobilization-induced atrophy is proteasome dependent. The proteasome has been demonstrated to be responsible for the bulk of the accelerated proteolysis in skeletal muscle during conditions of atrophy (46). To better define the proteolytic systems potentially responsible for atrophy during immobilization and to determine if inhibition of proteasome function could potentially rescue weight loss, the peptide boronate Velcade was administered in vivo. Gastrocnemius wet weight was not significantly different between saline- and Velcade-treated contralateral control muscle. Critically, injection of the proteasome inhibitor prevented ~53% of the wet weight loss of the immobilized muscle relative to the saline-treated contralateral control muscle (Fig. 8).

DISCUSSION

The accepted progression of disuse atrophy is rooted in adaptations in protein metabolism that involve both a reduction in synthesis and an increase in degradation. To investigate the signaling pathways likely to underlie these changes, we performed a systematic analysis of mediators of translational
initiation and elongation and components of the ubiquitination process in a well-defined model of unilateral hindlimb casting. Five days of unilateral immobilization drastically reduced gastrocnemius wet weight relative to the contralateral control muscle, which was an outcome of consorted atrophy and growth failure. In stark contrast to results from previous investigations of disuse, we observed no defect in the status of or intracellular signaling to critical regulators of the translational machinery, including Akt, mTOR, S6K1, 4E-BP1, and eIF and eEF under basal and growth factor-stimulated conditions at day 5. In accord with these findings, there was no alteration in the binding behavior of eIF4E under basal and growth factor-stimulated conditions after a moderate duration of unloading is likely attributable to elevated proteolysis, independent of decreases in protein synthesis and responsiveness of hypertrophic signaling cascades, observations that represent a considerable departure from conclusions reached previously with other models of disuse, including hindlimb suspension and bilateral hindlimb immobilization.

Table 1. Effect of 5 days of hindlimb immobilization on gastrocnemius mixed-muscle protein synthesis, RNA content, and translational efficiency

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<th>Control + Saline</th>
<th>Control + IGF-1</th>
<th>Immobile + Saline</th>
<th>Immobile + IGF-1</th>
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<tr>
<td>Rate of mixed-muscle protein synthesis, nmol Phe/mg protein ( h^{-1} )</td>
<td>6.92±0.53</td>
<td>6.37±0.46</td>
<td>7.00±0.49</td>
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<td>Muscle RNA concentration, mg RNA/g wet wt</td>
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<td>Muscle RNA content, mg RNA/muscle</td>
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<td>Translational efficiency, nmol Phe/mg RNA ( h^{-1} )</td>
<td>1.051±96</td>
<td>1.031±96</td>
<td>1.015±66</td>
<td>1.032±129</td>
</tr>
</tbody>
</table>

Values are means ± SE. Averages for protein synthesis, RNA content, RNA concentration, and translational efficiency were calculated with \( n = 8–10 \) animals/group. *Statistically different from control + saline values (\( P < 0.05 \)).

Control + Saline | Control + IGF-1 | Immobile + Saline | Immobile + IGF-1 |
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<tbody>
<tr>
<td>Rate of mixed-muscle protein synthesis, nmol Phe/mg protein ( h^{-1} )</td>
<td>6.92±0.53</td>
<td>6.37±0.46</td>
<td>7.00±0.49</td>
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<tr>
<td>Muscle RNA concentration, mg RNA/g wet wt</td>
<td>1.23±0.06</td>
<td>1.09±0.05</td>
<td>1.13±0.03</td>
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<tr>
<td>Muscle RNA content, mg RNA/muscle</td>
<td>2.03±0.10</td>
<td>1.90±0.05</td>
<td>1.55±0.09</td>
</tr>
<tr>
<td>Translational efficiency, nmol Phe/mg RNA ( h^{-1} )</td>
<td>1.051±96</td>
<td>1.031±96</td>
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</tr>
</tbody>
</table>

Values are means ± SE. Averages for protein synthesis, RNA content, RNA concentration, and translational efficiency were calculated with \( n = 8–10 \) animals/group. *Statistically different from control + saline values (\( P < 0.05 \)).

Fig. 6. Effect of 1, 3, and 5 days immobilization on mRNA content of muscle atrophy F-box (MAFbx)/Atrogin-1 and muscle ring finger 1 (MuRF1). A, top: quantitation of MAFbx/Atrogin-1 mRNA. Changes for all transcript sizes were qualitatively similar; data are shown for the indicated transcript. The value for contralateral control gastrocnemius of rats subject to 1 day immobilization is set at 1.0 AU. A, bottom: representative autoradiographs of MAFbx/Atrogin-1 and 18S mRNA, respectively. B, top: quantitation of MuRF1 mRNA. The value for contralateral control gastrocnemius of rats subject to 1 day immobilization is set at 1.0 AU. Data are presented as a line graph of the mean ± SE for 10 animals per group. Means with different letters are statistically different from one another (\( P < 0.05 \)). B, bottom: representative autoradiographs of MuRF1 and 18S mRNA, respectively. Con, control; Imm, immobilized.
the model because of depression of overall animal growth that may occur (36, 48). However, the coordinate sampling of wet weight at multiple time points and use of an internal contralateral control allow us to plainly demonstrate in young rats that the secondary growth failure response of the gastrocnemius is a direct consequence of disuse and not a general response of the organism per se.

Several lines of evidence support the conclusion that a repression of protein synthesis is not necessary for the decline in gastrocnemius wet weight after 5 days of unilateral immobilization. First, total RNA concentration was preserved in the casted muscle. Accordingly, the capacity for translation appears to be coordinately regulated with tissue size under conditions of immobilization and cannot account for the wasted phenotype. Second, a thorough analysis of translational control factors failed to detect any abnormality in regulators tasked with determining global rates of translation and translation of specific species of mRNA (those with 5'-TOPs and highly structured untranslated regions). Third, intracellular signaling to and from several strategically located kinases that control protein synthetic responses, including PDK-1, Akt, and mTOR (as assessed by phosphorylation events of known downstream targets), was preserved in the casted gastrocnemius muscle. Fourth, and most important, a direct in vivo measurement of protein synthesis and translational efficiency after an overnight fast did not indicate any depression of either synthetic index between immobilized and control gastrocnemius.

In contrast to the results of the present study, in a bilateral hindlimb immobilization model, casting produced a rapid (within 6 h) and persistent (up to 7 days) decrease in rates of protein synthesis (6, 49). This discrepancy in the responses of protein synthesis and translational control pathways may be a
consequence of animal age. Our results were obtained with young, rapidly growing rats, whereas the response of adult animals (used in the aforementioned reports with bilateral immobilization) after unilateral immobilization may differ because of the slower rate of protein accretion in mature muscle relative to its younger counterpart. Alternatively, this incongruity may reflect a fundamental difference in the models (i.e., there is a decrease in synthesis with bilateral but not unilateral casting). There are little data to support the contention that fasting imposed here masked a protein synthetic defect in the immobilized limb. First, in other catabolic conditions, a reduction in protein synthesis is readily apparent in fasted animals, and, second, unilateral casting produced comparable changes in the phosphorylation of key regulators of translation (e.g., mTOR, 4E-BP1, S6K1, eIF2α, and eEF2) independently of prandial state (data not shown). Although the data presented here argue against the contribution of a defect in protein synthesis to the immobilization-induced atrophy at a time point where it has been observed in previous investigations, we cannot exclude the possibility that a transient depression of synthesis or defect in signaling through pathways of translational control occurred at an earlier time point. Furthermore, the global rate of protein synthesis determined here may not be representative of the modulation of any one specific protein. We cannot discount the possibility that the reduced synthesis of one or more key regulatory protein(s) may underlie the wasting observed here. Regardless, the results presented here indicate that defective signaling to regulators of initiation and/or elongation and a decrease in protein synthesis are not prerequisites for moderate duration disuse-mediated atrophy, despite the widely accepted dogma that states otherwise.

Three main observations support the conclusion that unilateral immobilization upregulates ubiquitin- and proteasome-dependent protein degradation. First, unilateral immobilization increased the mRNA for several components of the ubiquitin-proteasome system, including polyubiquitin and the E3 ligases MAFbx/Atrogin-1 and MuRF1. This response of the polyubiquitin gene is an archetypal marker of proteolytic conditions (including cancer cachexia, denervation, dexamethasone injection, fasting, hindlimb suspension, and sepsis), changes coordinately with rates of ATP-dependent proteolysis (2, 34, 45, 47). Both of the E3s are expressed exclusively in cardiac and skeletal muscle where MAFbx/Atrogin-1 acts as the F-box component of a Skp1-Cullin/Cdc53-F-box ubiquitin ligase (4, 22), whereas MuRF1 is one member of a subset of RING finger proteins (9). The overall pattern of gene expression for both ligases is virtually identical to results obtained in the gastrocnemius after external fixation and similar to those seen with hindlimb suspension for a comparable time course (4). Second, the corroborating increases in ubiquitin immunoreactivity in muscle homogenates of unilaterally immobilized skeletal muscle are comparable to results obtained by immunoblot analysis in other well-characterized catabolic states (2, 16, 47, 52). Although these data do not represent a direct measurement of conjugating activity, they nevertheless support the notion that unilateral immobilization induces the ubiquitinylilation of muscle proteins and argues for involvement of energy-dependent proteolysis by the proteasome during hindlimb casting. Finally, the injection of Velcade (also known as PS-341 or bortezomib; see Ref. 1) prevented a significant loss of gastrocnemius wet weight after 3 days of immobilization, thereby directly implicating the proteasome in catalyzing a portion of the muscle wasting occurring in this model of disuse. Although not a direct measurement of proteolysis, the anti-catabolic effect of this proteasome inhibitor provides strong physiological evidence that increased protein degradation engenders significant gastrocnemius weight loss with immobilization.

Peak mRNA expression for polyubiquitin, MAFbx/Atrogin-1, and MuRF1 corresponds to the apparent point of transition between atrophy and growth failure, as indicated by muscle wet weight. We favor a model in which the dramatic upregulation of the E3 ligases during the early stages of immobilization causes gastrocnemius atrophy, whereas their decline to a lower constitutive expression at later time points induces growth failure. The significant role these proteins possess in control of muscle size has been demonstrated before with in vivo transgenic studies in both the heart and skeletal muscle; mice with a heart-specific overexpression of MAFbx/Atrogin-1 are resistant to cardiac hypertrophy induced by aortic banding (32), whereas in skeletal muscle a single gene deletion of MAFbx/Atrogin-1 or MuRF1 bestows on the muscle a robust resistance to denervation-induced atrophy (4). Altogether, these coordinated changes in gene expression are consistent with upregulated ubiquitin conjugation and consequent proteasomal targeting of skeletal muscle protein as major causal factors in mediating immobilization-induced atrophy.

The catabolic role of glucocorticoids is well supported by in vivo reports using adrenalectomized rats or administration of the type II glucocorticoid receptor antagonist RU-486 (35, 47), treatments that have a muscle-sparing effect. Critically, RU-486 treatment averted MAFbx/Atrogin-1 and MuRF1 transcription induced by experimental sepsis (53). In our study, RU-486 prevented neither immobilization-induced gastrocnemius atrophy and growth failure nor the upregulation of MAFbx/Atrogin-1 and MuRF1 at any time point. These results are consistent with prior investigations that found that adrenalectomized rats or those treated with RU-486 at doses greater than used here had only a minor sparing in unloading-induced atrophy of the soleus (25, 48). Although glucocorticoids are necessary for MAFbx/Atrogin-1 and MuRF1 induction in some catabolic states, they do not appear to be so in unilateral immobilization.

Although one published study has administered the proteasome inhibitor PSI to prevent acute sepsis-induced protein degradation and 3-methylhistidine release measured in vitro (16), to our knowledge, the data here represent the first demonstration of the ability of a proteasome inhibitor to attenuate in vivo muscle wasting resulting from a catabolic state. The reduction in muscle catabolism apparent with Velcade treatment is striking, yet this antagonism was not complete. This raises the possibility that other proteolytic pathways, such as the lysosomal or calcium-dependent systems, are responsible for the remaining loss in gastrocnemius weight. Furthermore, the relative contributions of energy-dependent vs. energy-independent proteolysis by the proteasome cannot be determined with this approach. The sparing effect reported here is likely to be underreported because of the comparison with the contralateral control muscle, which continues to grow throughout the 3-day period of immobilization. Moreover, we cannot exclude the possibility that a greater dose of Velcade could have a more pronounced effect. Nevertheless, these data pro-
vide proof of principle that the proteasome is responsible for increased muscle loss during unilateral immobilization.

In conclusion, we present here a model apparatus for the study of disuse muscle wasting using unilateral hindlimb immobilization. In the gastrocnemius, the prominent loss of muscle mass after a moderate duration of unloading results from coordinated atrophy and growth failure that is independent of a decrease in protein synthesis and appears at least partially contingent upon an increase in protein degradation. Mechanistically, the implementation of a defined transcriptional program, by a stimulus other than glucocorticoids, may accelerate proteolysis in the unloaded state. This response induces polyubiquitin, MAPbx/Atrogin-1, and MuRF1 gene expression and results in increased ubiquitin-conjugate formation in the wasted gastrocnemius. Antagonism of proteasome-dependent proteolysis averts a portion of skeletal muscle loss, an observation consistent with the role of the ubiquitin-proteasome system as a primary effector of immobilization-induced skeletal muscle atrophy.

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