

Temporal alterations in protein signaling cascades during recovery from muscle atrophy

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Childs, Thomas E., Espen E. Spangenburg, Dharmesh R. Vyas, and Frank W. Booth. Temporal alterations in protein signaling cascades during recovery from muscle atrophy. *Am J Physiol Cell Physiol* 285: C391–C398, 2003. First published April 23, 2003; 10.1152/ajpcell.00478.2002.—Currently, the repertoire of cellular and molecular pathways that control skeletal muscle atrophy and hypertrophy are not well defined. It is possible that intracellular regulatory signaling pathways are active at different times during the muscle hypertrophy process. The hypothesis of the given experiments was that cellular signals related to protein translation would be active at early time points of skeletal muscle regrowth, whereas transcriptional signals would be active at later time points of skeletal muscle regrowth. The phosphorylation status of p38 MAPK and JNK increased at the end of limb immobilization but returned to control values at recovery *day 3*. Transient increases in phosphorylation and in protein concentration occurred during recovery of soleus muscle mass. Phosphorylation of Akt, p70^{S6k}, and signal transducer and activator of transcription 3 (STAT3) peaked on recovery *day 3* compared with *day 0*. Glycogen synthase kinase (GSK)-3 β phosphorylation was increased on the sixth and fifteenth recovery day. In addition, transient peaks in seven protein concentrations occurred at different times of recovery: STAT3, calcineurin A (CaNA), CaNB, and β 4E-BP1 protein concentrations peaked on the third recovery day; p70^{S6k}, STAT3, Akt, and GSK3- β peaked on the sixth recovery day; and GSK3- β peaked on the fifteenth recovery day. The apexes of STAT3 and GSK3- β protein concentrations remained elevated for two recovery time points. Thus the time course of increase in molecules of signaling pathways differed as the young rat soleus muscle regrew from an atrophied state.

skeletal muscle; hypertrophy; rehabilitation; atrophy

HYPERTROPHIC GROWTH OF SKELETAL MUSCLE is an adaptive response to increases in mechanical load on the muscle. Currently, the repertoire of cellular and molecular pathways that control muscle atrophy and hypertrophy are incompletely defined. In fact, upon examination of the literature, it is readily apparent that there is little agreement on which signaling mechanisms play a major role in the hypertrophy process. For example, Dunn et al. (11) found that inhibition of calcineurin with cyclosporin A inhibited muscle hypertrophy after

4 wk of increased loading in mice. However, Bodine et al. (4) were unable to confirm these results, in that they found that inhibition of calcineurin with cyclosporin A did not have any affect on muscle hypertrophy in rats. Interestingly, multiple other signaling pathways have been suggested to play a role in activation and control of skeletal muscle hypertrophy (3, 14, 17, 18, 23, 32, 35). Therefore, it is possible that multiple pathways may contribute to regulation of skeletal muscle growth and that the activation of these pathways may occur at different times.

Generally, increases in the translation of mixed proteins will occur before increases in specific structural or metabolic mRNAs during exercise-induced muscle growth (see Ref. 8). Consequently, the hypothesis was tested that alterations in molecules of signaling pathways identified in the regulation of protein translation would be present during early time points of skeletal muscle regrowth, whereas signaling events for transcriptional signals would be activated later in the regrowth process. Therefore, we sought to explore the activation of signaling pathways that occurs during intermediate stages of the regrowth process by utilizing a model in which atrophy of the soleus muscle of the rat is induced by immobilization of the hindlimbs. After 10 days of immobilization, the restrictive stimulus was removed and the animals were allowed to freely ambulate around the cage. In previous studies using this model, normal ambulation has been shown to be an adequate stimulus for soleus muscle recovery to preatrophic size (6). At various time points, the soleus muscle was removed and activation of various signaling pathways thought to be important in the hypertrophic process was determined.

METHODS

Animals and hindlimb immobilization. Forty-one male, F1 generation, Fischer344 \times Brown Norway (FBN) rats that were 3 mo of age were obtained from National Institutes of Health (Harlan, Indianapolis, IN) and acclimatized for 2 wk before the study. Rats were housed during the immobilization and recovery phases with a 12:12-h light-dark cycle with two rats per cage. Experimental protocols had been approved by the Animal Use Committee of the University of Missouri, Columbia.

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Hindlimb immobilization. The hindlimb immobilization of the rats was performed according to the previously described procedures (7). Rats were lightly anesthetized with an intraperitoneal injection of ketamine (50 mg/kg) and acepromazine (5mg/kg) for attachment of the casting material. The hindlimbs of the animals were fixed in a shortened position with plaster casts as described previously (5). The animals were then checked daily for damage to casting material, which was subsequently repaired as necessary. After 10 days, the animals were anesthetized again, as described above, and the casting material of the recovery groups was removed completely and the animals were returned to their cages. No casting material was removed from the atrophy-only group until the animals were completely sedated to ensure no weight bearing occurred in this group. The anesthetic dosage per kilogram of body weight for dissection and death was ketamine (75 mg), xylazine (3 mg), and acepromazine (5 mg). At the time of death, the soleus muscle was carefully excised, weighed, and frozen in liquid nitrogen.

Protein isolation and concentration. Muscle tissue was prepared by glass on glass homogenization on ice in 50 mM HEPES (pH 7.4), 0.1% Triton-X, 4 mM EGTA, 10 mM EDTA, 15 mM $\text{Na}_4\text{P}_2\text{O}_7 \cdot \text{H}_2\text{O}$, 100 mM β -glycerophosphate, 25 mM NaF, 50 $\mu\text{g/ml}$ leupeptin, 50 $\mu\text{g/ml}$ pepstatin, 40.4 $\mu\text{g/ml}$ aprotinin, and 1 mM Na_3VO_4 . After homogenization, the samples were stored in aliquots at -80°C . The protein concentration of the samples was determined in triplicate via the Bradford procedure (Bio-Rad Protein Assay, Hercules, CA).

SDS-PAGE, Western blotting, and immunodetection. Protein samples were solubilized at a concentration of 1.25 mg/ml in loading buffer (62.5 mM Tris·HCl, pH 6.8, 20% glycerol, 2% SDS, 5% β -mercaptoethanol, and 0.025% bromophenol blue) and boiled for 5 min. Total protein was then loaded (100 $\mu\text{g/lane}$) onto 10% SDS-PAGE gels with these exceptions [25 μg protein/lane for glycogen synthase kinase (GSK)-3 β and 15% SDS-PAGE gels for 4E-BP1 and calcineurin B (CaNB)]. Separated proteins were then transferred onto nitrocellulose membranes (Osmonics, Westborough, MA) at 51 V for 2.5 h at 4°C in transfer buffer (25 mM Tris-base, 192 mM glycine, and 20% methanol). To verify transfer of proteins and equal loading of lanes, which occurred in all cases, the membranes were stained with Ponceau S. For immunodetection, membranes were blocked at room temperature in blocking buffer [5% nonfat dry milk in Tris-buffered saline (TBS)-T (0.1% Tween 20)] for 1 h, serially washed in TBS-T, and incubated with primary antibody in dilution buffer (5% BSA in TBS-T) overnight at 4°C . After another serial wash with TBS-T, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody and anti-biotin antibody in blocking buffer for 1 h, followed by another serial wash with TBS-T. With the use of enhanced chemiluminescence reagent (Perkin Elmer Life Sciences, Boston, MA), the HRP activity was detected with exposure to Kodak-XAR5 autoradiographic film for the appropriate durations to keep the integrated optical densities (IODs) within a linear and nonsaturated range for all bands of each gel. The IODs were quantified using ImageQuant densitometry software (Molecular Dynamics, renamed Amersham Biosciences, Sunnyvale, CA). To correct the sample IODs for exposure time differences, the same quantity of control muscle sample was loaded on every gel.

Antibodies. The primary antibodies phosphoSer⁴⁷³-Akt (1:500 dilution), Akt (1:1,000), phospho-Ser⁹-glycogen synthase-3 β (GSK-3 β) (1:1,000), phospho-Thr²⁰²/Tyr²⁰⁴ p44/42 mitogen-activated protein kinase (p44/42 MAPK) (1:1,000), p44/42 MAPK (1:1,000), phospho-Thr¹⁸⁰/Tyr¹⁸²-p38 MAPK (1:1,000), p38 MAPK (1:1,000), phospho-Thr¹⁸³/Tyr¹⁸⁵-

stress-activated protein kinase/Janus kinase (SAPK/JNK) (1:500), SAPK/JNK (1:1,000), phospho-Thr³⁸⁹-p70 S6 kinase (p70^{S6k}) (1:500), p70^{S6k} (1:500), 4E-BP1 (1:1,000), phospho-Tyr⁷⁰⁵-signal transducer and activator of transcription 3 (STAT3) (1:1,000), STAT3 (1:1,000), and anti-biotin antibody (1:4,000) were purchased through Cell Signaling Technology (Beverly, MA). The antibody for GSK-3 β (1:2,500 dilution) was purchased through Transduction Laboratories (Lexington, KY). Antibodies calcineurin A (1:10,000) and CaNB (1:2,000) were purchased through Sigma-Aldrich (St. Louis, MO) and ABR (Golden, CO). Anti-rabbit and anti-mouse secondary antibodies (1:7,500) were purchased from Amersham Biosciences (Piscataway, NJ).

Statistical analysis. All muscle mass data were analyzed using a one-way analysis of variance and a Tukey's post hoc test ($P < 0.05$). Not all protein data showed normality and equal variance, so Western data were analyzed utilizing the nonparametric Kruskal-Wallis test, and post hoc analysis was performed using a Dunn's multiple range test with $P < 0.01$ designated as statistically significant.

RESULTS

Decrease in soleus weight after hindlimb immobilization. After 10 days of immobilization, the soleus muscle mass significantly decreased by 38.5% from control (Fig. 1). The soleus muscle of the 3-day recovery group was significantly less than controls at the start of immobilization (having only regained 23.5% of lost soleus mass); the 6-day recovery group regained 37% of lost mass, and its mass was not significantly different from the preimmobilization controls.

Change in phosphorylation levels and total protein expression of various signaling proteins in atrophied and regrowing soleus muscle. Phosphorylation of p38 was significantly reduced 98% in all recovery time points from its value at 10 days of immobilization (Fig. 2A). JNK phosphorylation was significantly reduced by 84 and 99% in the recovery time points of 3 and 15 days, respectively, from the 10-day immobilization group (Fig. 2B).

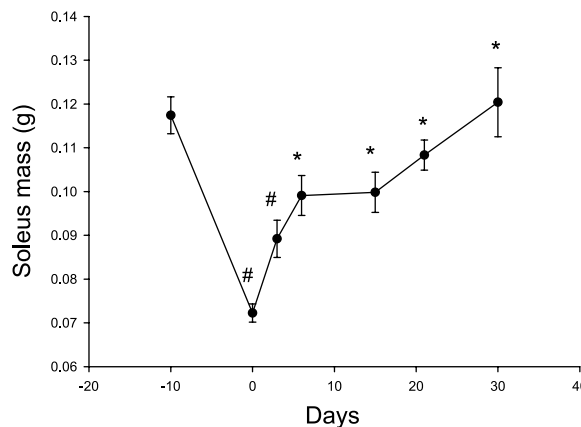


Fig. 1. Changes in soleus muscle mass after 10 days of hindlimb immobilization, followed by varying days of voluntary ambulatory recovery of the rats. #Statistical difference ($P < 0.05$) from the control group. *Statistical difference ($P < 0.05$) from the 10-day-immobilized groups. The 21- and 30-day recovery masses of soleus muscle are shown to indicate that recovery time points until 15 days represent the first half of muscle regrowth.

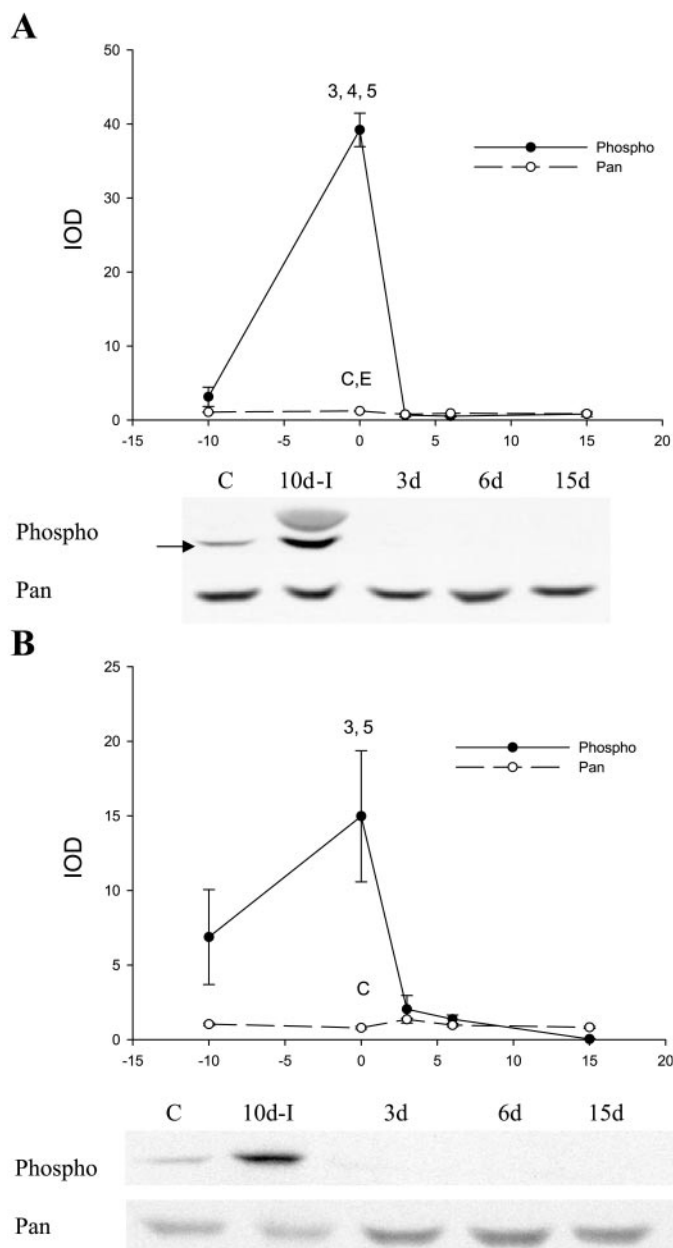


Fig. 2. Alterations in the phosphorylation status and concentration of proteins linked with stress signaling pathways during muscle atrophy and recovery from muscle atrophy. A: p38; B: JNK. Integrated optical densities (IOD) are shown on the y-axis. The time line (days) of the experimental design is shown on the x-axis. Control is -10 days. *Day 0* is the last day of the 10-day hindlimb immobilization. For statistical analysis, *day 0* was designated as the reference time point. Recovery days from the 10 days of immobilization are 3, 6, and 15 days. For all data concerning phosphorylation status (●—●), statistical differences ($P < 0.01$) are symbolized as follows: 1, difference from the control group; 3, difference from the 3-day group; 4, difference from the 6-day group; and 5, difference from the 15-day group. For all data concerning the total concentration of the signaling protein (pan; ○—○), statistical differences ($P < 0.01$) are symbolized as follows: A, difference from the control group; C, difference from the 3-day group; D, difference from the 6-day group; and E, difference from the 15-day group. Representative blots for each specific target are depicted below respective graph, with the phosphorylation status blot on top and the total protein blot (pan) on the bottom.

Expression levels of phosphorylated Akt decreased significantly by 55 and 59% after 10 days of immobilization, compared with the control and 3-day recovery time points, respectively (Fig. 3A). There was a significant increase in Akt protein concentration by 37% at the sixth day of recovery, compared with the 10-day immobilization group.

The phosphorylation of p70^{S6k} was significantly increased by 12- and 21-fold at *day 3* of recovery compared with the control and 10-day immobilized groups, respectively (Fig. 3B). p70^{S6k} protein concentration was significantly higher by 102% at *day 6* of recovery compared with the 10-day immobilized group.

4E-BP1 phosphorylation was estimated by determination of the protein concentration of the α , β , and γ forms of 4E-BP1 (Fig. 3C). According to previous work (23, 29), α 4E-BP1 exhibits the least amount of phosphorylation and therefore migrates the fastest, whereas the γ form is the most phosphorylated and migrates the slowest, and finally the β form exhibits an intermediate amount of phosphorylation and therefore migrates between the α and γ forms. Significant increases in the β 4E-BP1 form [calculated as $(\alpha + \beta)/\alpha$] were detected in the 3-day recovery compared with the 10-day immobilization (Fig. 3C). In addition, significant increases in the γ 4E-BP1 [calculated as $(\alpha + \gamma)/\alpha$] were detected in the 10-day immobilization group compared with the control.

Phosphorylated GSK-3 β expression was significantly increased by 318 and 261% on *days 6* and *15*, respectively, compared with the 10-day-immobilized group (Fig. 4). Also, GSK-3 β protein concentration was significantly increased by 102 and 105% on *days 6* and *15*, respectively, compared with the immobilized group.

STAT3 phosphorylation was 751 and 259% higher in the 3- and 6-day recovery groups, respectively, compared with the 15-day recovery group (Fig. 5A). Total STAT3 protein expression was significantly increased by 253 and 280% in the 3- and 6-day recovery groups, respectively, compared with the 10-day immobilization group.

Calcineurin A (CaNA) protein concentration was significantly elevated in the 3-day recovery group compared with the 10-day-immobilized group by 110% (Fig. 5B). CaNB subunit protein concentration was significantly increased in the 3-day recovery compared with the 10-day-immobilized group by 105% (Fig. 5C).

No changes in p44/42 MAPK (ERK1 or 2) phosphorylation status were detected at any time point in immobilized group or any recovery groups (data not shown).

DISCUSSION

During regrowth from skeletal muscle atrophy, we found that cellular signaling pathways associated with protein translation were increased during the early time points of regrowth, whereas cellular signaling pathways associated with increased gene transcription and translation were activated during later time points of muscle regrowth. On the basis of previous research

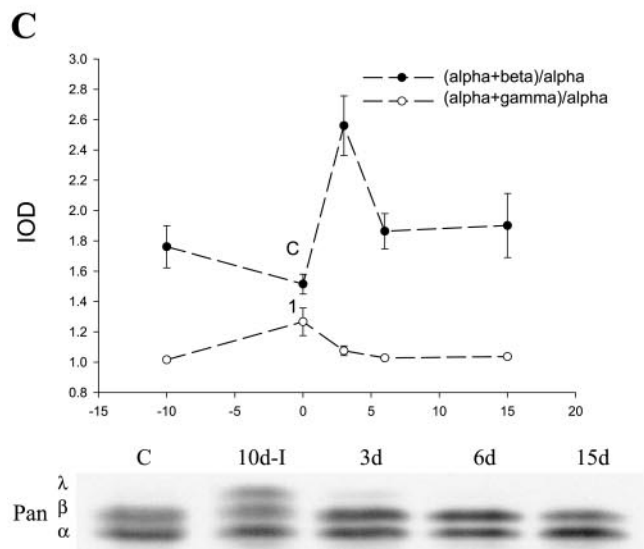
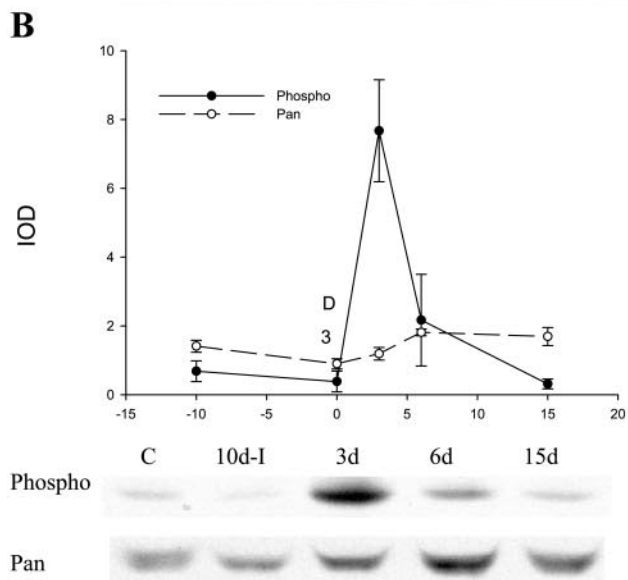
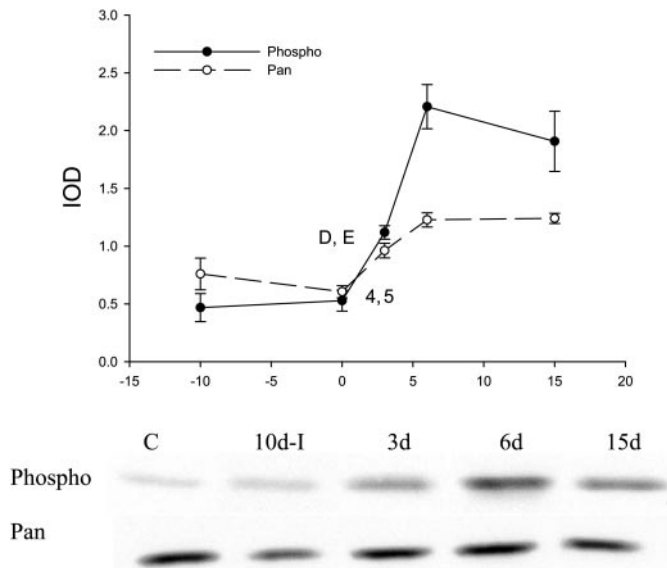
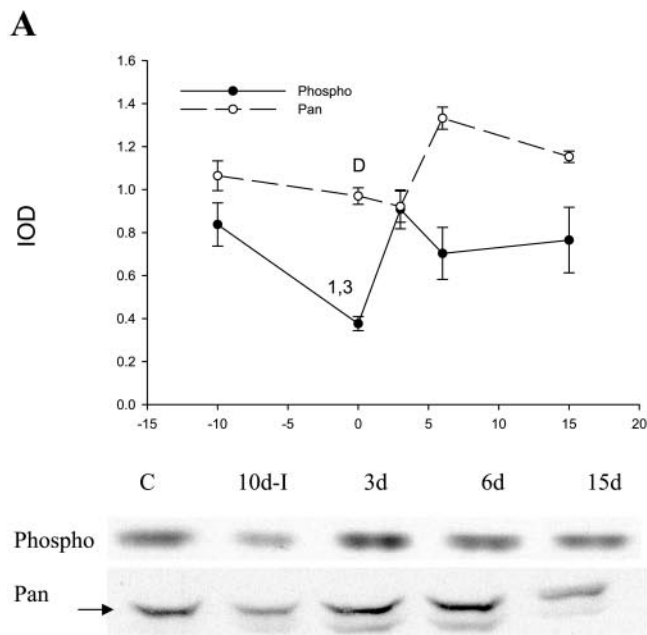
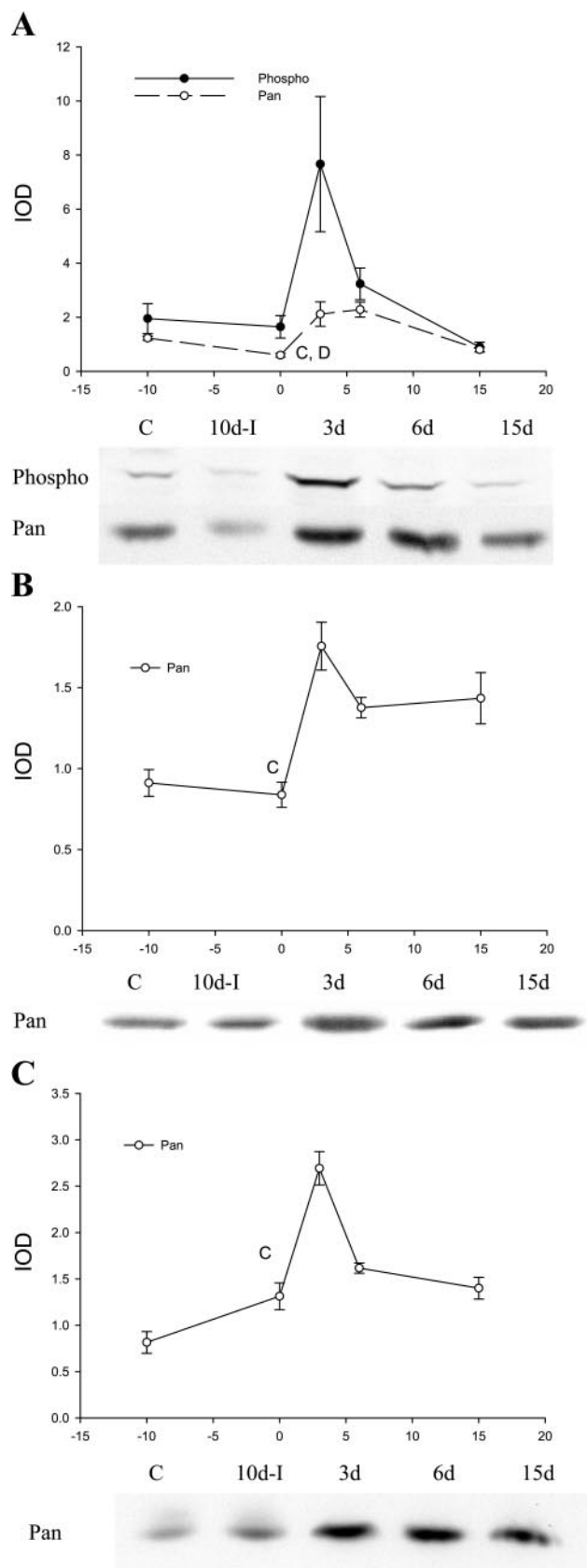


Fig. 4. Alterations in the phosphorylation status and concentration of glycogen synthase kinase (GSK)-3 β , a protein linked with gene transcription and protein translation signaling during muscle atrophy and recovery from muscle atrophy. The format of the presentation of the data is exactly the same as described in Fig. 2.

and the above finding, we confirm our initial hypothesis, in that signaling pathway activation during regrowth is based on important time constants involving changes in skeletal muscle protein translation or gene transcription (Fig. 6).

Phosphorylated p38 MAPK and p38 MAPK protein concentration were significantly elevated after the 10 days of immobilization and subsequently returned to control in the 15-day recovery period. Hunter et al. (21) found no change in p38 activity and concluded that cytokine-mediated pathways were not activated in the rat soleus muscle on the seventh day of hindlimb unloading. Possibly limb immobilization, which decreases EMG activity to 5–15% of control (13), activates cytokine signaling pathways, whereas unloading does not increase p38 activity, because no EMG reductions (2) occur at the 7-day time point. Interestingly, JNK followed a trend similar to that of p38, in that the highest expression levels were found at the end of the immobilization period and the expression levels subsequently returned to control during the recovery process. A JNK1-mediated signal cascade has been shown to contribute to the progression of the disease pathogenesis in dystrophic muscle (24). Because environmental stresses and inflammatory cytokines usually simultaneously activate both p38 MAPK and JNK (25), one interpretation may be that regrowth of the soleus muscle removes a stressor associated with inactivity-induced muscle atrophy. An alternative interpretation

Fig. 3. Alterations in the phosphorylation status and concentration of proteins linked with protein translation signaling during muscle atrophy and recovery from muscle atrophy. A: Akt; B: p70^{S6k}; C: 4E-BP1. The format of the presentation of the data is exactly the same as described in Fig. 2.



is based on p38 MAPK overexpression in a transgenic heart. Constitutive activation of p38 MAPK produced interstitial fibrosis, expression of fetal marker genes characteristic of cardiac failure, no significant hypertrophy at the organ level, and premature death at 7–9 wk (27). Therefore, the possibility exists that p38 and JNK activation plays a role in skeletal muscle atrophy during hindlimb immobilization.

The increases in Akt and p70^{S6k} phosphorylation and in β 4E-BP1 in the soleus muscle at the third day of recovery from 10 days of immobilization-induced atrophy suggest their potential contribution to increasing protein translation and muscle mass (Fig. 3C). This confirms a portion of our hypothesis that signaling related to translation would increase during early time points of muscle regrowth after ending immobilization. Others have reported that during the early phases of increased muscle loading, there are subsequent increases in insulin-like growth factor-1 (IGF-I) protein expression (1). Akt, p70^{S6k}, and 4E-BP1 are all known to be activated by increases in IGF-I (26, 36) concentration, so it is not unreasonable to suggest that early activation of signaling proteins associated with protein translation may be at least partially mediated by increases in IGF-I concentration associated with increased muscle loading.

The phosphospecific antibody employed on the third recovery day showed an increased p70^{S6k} Thr³⁸⁹ phosphorylation that others have found necessary for complete enzymatic activation of p70^{S6k}-specific kinase activity (16). Previously, an increase in p70^{S6k} phosphorylation in skeletal muscles 3–36 h after exercise correlated tightly with the percent change in muscle mass after 6 wk of eccentric-type training against an unloaded antagonistic muscles (3). However, in the current study, p70^{S6k} phosphorylation and p70^{S6k} protein returned from their higher values to control levels on the sixth and the fifteenth days, respectively, of daily voluntary cage activity after limb immobilization, time points that were not examined in the aforementioned eccentric training study (3). Furthermore, Bodine et al. (4) found that a muscle injection of a constitutively active p70^{S6k} induced significant increases in skeletal muscle fiber size in normal and denervated muscle fibers. Thus increased phosphorylation of p70^{S6k} likely plays some role in initiating the regrowth process, but in the regrowth model p70^{S6k} phosphorylation does not appear to play a role in the continuance of soleus muscle regrowth 6–15 days after immobilization ended.

Interestingly, previous reports have suggested that p70^{S6k} phosphorylation may be regulated by an upstream kinase, termed protein kinase B or Akt, under specific conditions (36). Here, we found increases in p70^{S6k} Thr³⁸⁹-phosphorylation and Akt Ser⁴⁷³-phos-

Fig. 5. Alterations in the phosphorylation status and concentration of proteins linked with gene transcriptional signaling during muscle atrophy and recovery from muscle atrophy. A; STAT3; B; calcineurin A; C; calcineurin B. The format of the presentation of the data is exactly the same as described in Fig. 2.

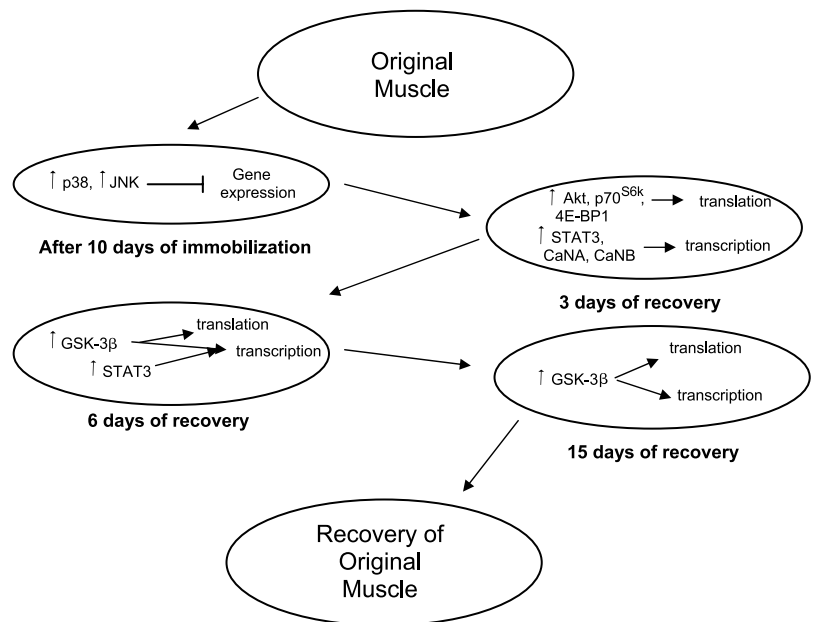


Fig. 6. Schematic of the identified changes in signaling molecules in the soleus muscle that were identified in a portion of the cascade of events leading to net protein balance after 10 days of hindlimb immobilization and during the subsequent 10-day recovery.

phorylation occurring from recovery *days 0* to 3, suggesting that Akt may be signaling p70^{S6k} phosphorylation. The lack of a prolonged activation of Akt differs from the findings of Bodine et al. (4), who found that Akt was in fact phosphorylated in the functional overload model at 3, 7, and 14 days, which could be explainable by the more extreme nature of muscle loading in the functional overload model compared the regrowth model.

Alteration in the phosphorylation status of 4E-BP1 also transiently occurred at the third recovery day. 4E-BP1 has been shown to be resolved into multiple electrophoretic forms termed α , β , and γ , representing progressively increased phosphorylated forms (29). In addition, 4E-BP1 is thought to be activated via Akt and mTOR (23). Hypophosphorylated α 4E-BP1 binds to eIF4E, sequestering this protein in an inactive 4E-BP1·eIF4E complex. Full phosphorylation of 4E-BP1, the γ form, regulates translation initiation and protein synthesis by inducing the dissociation of the 4E-BP1·eIF4E complex. β 4E-BP1 increased 147% on recovery *day 3* compared with the 10-day-immobilized value, which suggests that stimulation of the translational initiation of protein synthesis may occur early in the regrowth process. The possible early activation of protein synthesis in the immobilization-recovery model would be in agreement with the conclusion made by Kimball et al. (23), who stated that a consistent finding, despite markedly different experimental models of exercise, is that muscle protein synthesis is elevated after acute resistance exercise with no changes in muscle RNA. Thus signaling via Akt, p70^{S6k}, and 4E-BP1 seems to be early in the regrowth process and may be related to a stimulation of mRNA translation.

Although γ 4E-BP increased 15-fold at the end of the 10 days of immobilization, γ 4E-BP was barely detectable in the control condition. It is notable that the only time point in which γ 4E-BP was visibly detectable was

after the 10 days of immobilization. As protein synthesis rates are decreased in atrophying immobilized muscles (34) and muscles from immobilized limbs have no alteration in insulin sensitivity for protein synthesis (9), the increase in phosphorylated γ 4E-BP1 seems paradoxical. Potentially, other factors than the regulation of mRNA translation are rate limiting for protein synthesis in soleus muscle atrophy at the tenth day of immobilization, such as eIF2B (23), or, as shown, p70^{S6k} phosphorylation was not increased at this time (Fig. 3B).

On the third and sixth recovery days, STAT3 phosphorylation at tyrosine residue 705 and protein concentration were significantly increased compared with the 15-day recovery. STAT3 is a transcription factor phosphorylated by JAK2 in response to elevations in concentration of various growth factors such as growth hormone (GH) (20) or leukemia inhibitory factor (LIF) (32). GH exerts many of its physiological functions by regulating the transcription of genes for a variety of proteins, including IGF-1 (20), which increases in hypertrophying skeletal muscle (37). GH also increases the expression of IGF-I mRNA in C₂C₁₂ myoblasts (15), which would amplify the growth effect (1). LIF activates satellite cell proliferation, the time course proliferation of which in other skeletal muscle growth models (30) is coincident with increased STAT3 activation (32). Thus the increase in STAT3 could play a role in soleus muscle regrowth.

Mitchell et al. (28) concluded that published reports provide a controversial role for CaN (also called protein phosphatase 2B) in regulating skeletal muscle growth. CaN is a serine/threonine protein phosphatase and the only known phosphatase activated by Ca²⁺ and calmodulin (CaM). Its 58- to 64-kDa catalytic subunit (CaNA) contains specific domains with regulatory functions, including an autoinhibitory domain near the carboxy terminus, a CaM-binding domain, and a bind-

ing domain of a regulatory subunit, CaNB (31). According to Shibasaki et al. (31), Ca^{2+} binding to CaNB plays a structural rather than a regulatory role by allosterically activating CaNA through conformational folding. In the current experiment, the protein contents of CaNA and CaNB increased only on recovery day 3. Dunn et al. (12) concluded that unless significantly compromised, the endogenous pool of calcineurin accommodates the signaling requirements related to extremes in functional demand in type II muscle, but they did not examine type I muscle as used in the current study. A fiber type differential in endogenous calcineurin expression exists (28, 33). CaNA is expressed at higher levels in skeletal muscles dominated by fast fiber expression (33), whereas CaNB protein expression is higher in muscles composed mostly of slow fibers (28). The transient increases in both CaNA and CaNB proteins in the regrowing soleus muscle thus suggest an additional regulatory mechanism for CaNA and CaNB expression other than fiber type, which confirms the previous observations of Spangenburg et al. (33). Our current observations confirm Mitchell et al.'s (28) statement that CaN-mediated signaling pathways in skeletal muscle may be regulated not only by inductive signals that increase intracellular Ca^{2+} but also via a complex mechanism involving regulation of expression of CaN subunits.

The increase in GSK-3 β phosphorylation and protein at the sixth and fifteenth days of recovery is intriguing due to GSK-3 β 's reported role as a negative regulator of protein translation and gene expression (19). Phosphorylation of the serine 9 residue of GSK-3 β inhibits its kinase activity, thereby maintaining NFAT transcriptional activity in C₂C₁₂ myotubes (35). In addition, activated (unphosphorylated) GSK-3 β plays a significant role in the regulation of protein synthesis, in that it has the ability to inhibit the activity of the protein translation initiation factor eIF2B (23). Inhibition of GSK-3 β by hypertrophic stimuli has been proposed to be an important mechanism contributing to the development of cardiac hypertrophy (19). Application of inhibitors of GSK-3 β activity (i.e., IGF-I or LiCl) produced significant increases in myotube size in culture (35). The associative increase in GSK-3 β phosphorylation status and the continued regrowth of the soleus muscle on recovery days 6 and 15 identifies a potential signal for continued muscle mass enlargement, confirming another part of our hypothesis that a longer-acting signal would affect transcriptional regulation. Because there was no change in Akt phosphorylation levels on recovery days 6 and 15 when GSK-3 β was phosphorylated, it is likely that a different upstream kinase is regulating GSK-3 β in the regrowing soleus muscle. GSK-3 β activity can be inhibited by at least eight molecules (Akt, Wnt, ILK, PKA, PKC δ , LiCl, p90^{RSK}, and p70^{S6k}) and activated by tyrosine kinase (19).

An intriguing observation was that seven protein concentrations significantly increased from values found in control or 10-day-immobilized groups with their peak values occurring at different recovery times

[four on the third recovery day (STAT3, CaNA, CaNB, and β 4E-BP1), three on the sixth recovery day (Akt, p70^{S6k}, STAT3, and GSK-3 β), and one on the fifteenth recovery day (GSK-3 β) (where the significant increases of STAT3 and GSK-3 β concentrations extended for two recovery time points)]. Thus alterations in signaling during muscle regrowth not only involve changes in phosphorylation status as suggested for CaN by Mitchell et al. (28) but also comprise increases in the protein concentrations for seven other signaling molecules. Thus sequential changes not only occur for phosphorylation of signaling proteins but also for concentration of proteins functioning in the signaling, suggesting multiple adaptive responses to restore the mass of the regrowing soleus muscle as it recovers from atrophy. The upstream signals coordinating the staggered transient increases of protein concentrations remain to be determined.

The experimental design for the current experiment does have limitations. Although the design allows for measurements of multiple signaling proteins that potentially could play some role in regrowth as atrophied soleus muscle recovers from hindlimb immobilization, the size of the soleus muscle limits selection of assays. To test a broad range of molecules for potential alterations during muscle regrowth, soleus muscle mass was only sufficient to perform Western analysis. In addition, arbitrary decisions were made as to the selection of recovery time points.

These data are the first to demonstrate the activation and deactivation of signaling pathways occurs in a specifically designed manner over an extended time course of muscle growth. Not only were crucial steps in multiple signaling pathways altered by phosphorylation and protein expression, but their transient and staggered nature is intriguing because it suggests that a response to a return of muscle usage after atrophy is orchestrated by sequential waves of increased molecules of signaling pathways as the young rat soleus muscle regrows from atrophy. An important prediction from the current observations is that future mapping of the integration among multiple signaling pathways, the activities of which wax and wane at different times during muscle regrowth from atrophy, could be a daunting task.

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DISCLOSURES

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