Skeletal muscle is a highly plastic tissue with the ability to adapt to changing functional demands (6). For example, increasing mechanical load induces skeletal muscle hypertrophy, whereas muscle inactivity results in muscle atrophy. Although the factors regulating muscle hypertrophy and atrophy continue to be investigated, the precise intracellular mechanisms responsible for these forms of muscle remodeling are not well understood (7).

It has been reported that growth factors and cytokines such as insulin-like growth factor I (IGF-I), fibroblast growth factor, interleukin-6, and leukemia inhibitory factor are associated with muscle hypertrophy (7, 11, 22). In particular, IGF-I has been shown to be one of the key factors involved in muscle hypertrophy. For example, increased levels of IGF-I, along with calmodulin and calcineurin (CaN), promote myotube growth in association with nuclear factor of activated T cells (NFAT) c1 (21). More specifically, recent studies suggest that the IGF-I-phosphatidylinositol 3′-kinase (PI3K)-protein kinase B [PKB, also called Akt (PKB/Akt)]-mammalian target of rapamycin (mTOR) pathway is important for mediating muscle hypertrophy, whereas downregulation of this pathway is associated with muscle atrophy (5, 27, 29). Collectively, these studies suggest that several of the downstream targets of IGF-I-mediated signal transduction play a significant role in the induction of skeletal muscle hypertrophy.

CaN, a Ca\(^{2+}\)/calmodulin-dependent protein serine/threonine phosphatase, may also be an important signaling molecule in skeletal muscle fiber growth and maintenance of the slow muscle fiber gene program (1, 3, 4, 9, 10, 12, 13, 15, 21, 25, 26, 30, 32, 33, 35). For example, injection of cyclosporin A (CsA) and FK-506, potent inhibitors of CaN activity, has been reported to prevent mechanical overloading-induced muscle hypertrophy (12). Moreover, mechanical overloading-induced fast-to-slow fiber type transformation can be prevented by inhibition of CaN activity via CsA and FK-506 (9). Indeed, a recent report indicates that increased expression of type I myosin heavy chain (MHC) in electrically stimulated regenerating soleus muscles can be blocked by CsA or FK-506 (33). Nonetheless, the role of CaN in the regulation of muscle fiber growth and slow fiber phenotype expression remains controversial, inasmuch as some studies failed to find a strong link between CaN and control of slow fiber phenotype (25, 35).

It is well known that hindlimb unloading (HLU), especially in the slow soleus muscle, promotes muscle atrophy and a shift in fiber phenotype from slow to fast fibers (i.e., type I to type II), and these changes can be reversed after recovery from HLU. However, limited information is available regarding the signal transduction pathways that participate in the recovery of muscle mass in response to disuse and increased muscle loading. These experiments investigated changes in signal transduction of the downstream pathways of PKB/Akt and CaN during recovery following disuse-induced muscle atrophy. A 10-day period of hindlimb unloading (HLU) via tail suspension (male rats) was used to produce soleus muscle atrophy. Muscle recovery was achieved by returning animals to normal ambulation for 3–10 days. HLU resulted in significant muscle atrophy and a slow-to-fast fiber transition as revealed by appearance of type IId/x and Iib myosin heavy chain (MHC) isoforms. Muscle mass in HLU animals recovered to control (Con) levels after 10 days of reloading, but the fast-to-slow shift in muscle MHC was incomplete, as indicated by the continued presence of type IId/x MHC. Ten days of HLU resulted in a significant decrease (−43%) in muscle levels of phosphorylated PKB/Akt. In contrast, muscle levels of phosphorylated PKB/Akt were greater (+56%) in HLU than in Con animals early after the onset of reloading (3 days). Soleus levels of phosphorylated p70S6K were significantly higher (+26%) in HLU animals after 3 days of muscle reloading. Muscle levels of phosphorylated PKB/Akt and phosphorylated p70S6K returned to Con levels by 10 days of recovery. Moreover, muscle CaN levels were significantly higher than Con levels after 10 days of muscle reloading. These findings are consistent with the hypothesis that PKB/Akt and its downstream mediators are active in the regrowth of muscle mass during the early periods of recovery from muscle atrophy. Our data support the concept that CaN is involved in muscle remodeling during the later phases of recovery from disuse muscle atrophy.
atrophyed muscle. Therefore, this study investigated time-dependent changes in signal transduction pathways involved in muscle regrowth and MHC isoform transition during the recovery period of atrophied soleus muscle. On the basis of previous reports and preliminary experiments in our laboratory, we hypothesized that the downstream pathway of PKB/Akt is downregulated in response to HLU but is reactivated early in the recovery period after HLU. We also postulated that CaN is not required for muscle regrowth during the early stages of muscle reloading but becomes elevated during the later stages of recovery from disuse muscle atrophy when fiber type changes are occurring.

MATERIALS AND METHODS

Animals. The experiments were approved by the Animal Use Committee of Yamaguchi University and followed the guiding principles for the care and use of animals of the Physiological Society of Japan. Male Wistar rats (n = 48, 7 wk of age) were randomly assigned to two primary groups: sedentary control (Con, n = 24) and hindlimb unweighting (HLU, n = 24). The HLU animals were further divided into three experimental groups: 1) HLU with no reloading of skeletal muscles (n = 8), 2) HLU with 3 days of reloading of skeletal muscles (n = 8), and 3) HLU with 10 days of reloading of skeletal muscles (n = 8). For each the three HLU experimental groups there was a separate and time-matched control group of animals (n = 8/group). The rats were individually housed in a climate-controlled room (26 ± 1°C, 56 ± 1% relative humidity, and 12:12-h light-dark photoperiod) and fed standard rat chow and water ad libitum. After 7 days of standard housing, the HLU group was exposed to HLU for 10 days as described previously (21). After release from unweighting, the hindlimb muscles of the rats were reloaded by a return to normal cage activity. Weight-matched sedentary rats served as control in each recovery period. Weight matching was achieved by using younger (control) animals in each of the control groups; these animals were ~7 days younger than their HLU counterparts. At appropriate times at the completion of the experiment, rats were anesthetized by administration of pentobarbital sodium (100 mg/kg body wt). When animals reached a surgical plane of anesthesia, soleus muscles of both legs were quickly removed, weighed, and then rapidly frozen in liquid nitrogen. Muscles were stored at ~80°C until analysis.

Muscle preparation. Samples were minced and homogenized in ice-cold homogenization buffer (0.1% Triton X-100, 50 mM HEPES, pH 7.4, 4 mM EGTA, 10 mM EDTA, 15 mM Na2HPO4, 100 mM β-glycerophosphate, 25 mM NaF, 5 mM Na3VO4, and leupeptin, pepstatin, and aprotinin at 50 mg/ml each). Homogenates were centrifuged at 12,000 g for 15 min at 4°C, and the protein concentration of the supernatants was determined in triplicate with a protein determination kit (Bio-Rad Laboratories, Richmond, CA).

SDS-PAGE, Western blotting, and immunodetection. Samples were solubilized in sample loading buffer (30% glycerol, 5% β-mercaptoethanol, 2.3% SDS, 62.5 mM Tris·HCl, pH 6.8, and 0.05% bromphenol blue) at 2 mg/ml and incubated at 60°C for 10 min. Proteins were then separated by 10% SDS-PAGE and subjected to Western blotting for 60 min at 4°C onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA) with use of a Bio-Rad mini trans-blot cell at a constant voltage of 100 V in transfer buffer (25 mM Tris·HCl, pH 8.3, 192 mM glycine, and 20% methanol). After protein transfer, the membranes were blocked for 1 h at room temperature in blocking buffer [5% nonfat dry milk in Tween-Tris-buffered saline (T-TBS: 20 mM Tris·HCl, 150 mM NaCl, and 0.05% Tween 20, pH 7.5)]. After serial washes with T-TBS, the membranes were incubated with primary antibodies to phosphorylated Ser473-Akt (diluted 1:1,000 in 5% BSA in T-TBS; Cell Signaling, Beverly, MA), phosphorylated Thr380-p70 S6 kinase (p70S6K, 1:1,000; Cell Signaling), phosphorylated Ser240/244-S6 ribosomal protein (1:1,000; Cell Signaling), or CaN (1:10,000; Sigma, St. Louis, MO) primary antibodies overnight at 4°C. After several washes in T-TBS, membranes were incubated with anti-rabbit or anti-mouse horseradish peroxidase-conjugated secondary antibodies (1:2,000 in blocking buffer; Cell Signaling) for 1 h at room temperature. ECL Plus reagents (Amersham Biosciences, Piscataway, NJ) were used to detect protein signals by ATTO Light Capture (Tokyo, Japan), and band intensities were quantified by densitometry.

Measurement of muscle water content. Total water content of muscle sections was determined using a freeze-drying technique incorporating a vacuum pump with a negative pressure of ~1 mmHg. The measurement was terminated when the same weight was recorded three times in succession during a 6-h interval.

Statistics. Values are means ± SE. Statistical significance in muscle mass and muscle-to-body weight ratios was determined using a two-way analysis of variance for multiple comparisons followed by Tukey’s post hoc test. Because of a failure to meet the requirement of a normal distribution, all data expressed as percentages were analyzed using a nonparametric (Kruskal-Wallis) test. P < 0.05 was considered significant.

RESULTS

Soleus muscle mass, soleus weight-to-body weight ratio, and water content in all experimental groups are shown in Table 1. Ten days of HLU resulted in a significant decrease (~36%) in soleus muscle weight. Furthermore, soleus muscle weights remained ~22% lower in HLU animals exposed to 3 days of reloading (recovery) than in Con animals. However, soleus muscle weights in HLU animals subjected to 10 days of muscle reloading did not differ from those in Con animals and were significantly higher than in animals exposed to 10 days of HLU and 3 days of recovery (Table 1). Finally, there were no differences in muscle water content between the experimental groups.

Compared with Con, soleus muscle levels of phosphorylated PKB/Akt were significantly decreased (~43%) after 10 days of HLU (Fig. 1). However, muscle levels of phosphorylated PKB/Akt tended to be greater (~56%) in HLU animals after 3 days of reloading than in Con animals. Moreover, muscle phosphorylated PKB/Akt levels were greater in animals in the 3- and 10-day recovery groups than in animals exposed to 10 days of HLU alone.

Figures 2 and 3 illustrate the levels of phosphorylated p70S6K and phosphorylated S6 ribosomal protein levels, respectively.

Table 1. Muscle mass, muscle weight-to-body weight ratio, and water content in soleus muscle after HLU and during 3–10 days of muscle reloading

<table>
<thead>
<tr>
<th></th>
<th>Day 0</th>
<th>Day 3</th>
<th>Day 10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Muscle wt, mg</td>
<td>110.2±2.2</td>
<td>112.0±2.2</td>
<td>134.4±4.0</td>
</tr>
<tr>
<td>Relative muscle wt, mg/kg body wt</td>
<td>69.8±3.4±</td>
<td>86.9±4.8±</td>
<td>126.7±6.2</td>
</tr>
<tr>
<td>Water content, %</td>
<td>373.4±6.7</td>
<td>379.6±6.8</td>
<td>398.9±11.6</td>
</tr>
<tr>
<td>HLU</td>
<td>230.5±11.2±</td>
<td>288.9±18.7±</td>
<td>373.6±15.6</td>
</tr>
</tbody>
</table>

Values are means ± SE. Relative muscle mass is soleus weight-to-body weight ratio. Water content is expressed as a percentage of total muscle mass.

*Significantly different from control, P < 0.05. †Significantly different from HLU at day 10, P < 0.05.
spectively, in the soleus muscle across the experimental groups. Phosphorylated p70S6K levels were significantly greater in HLU animals at day 3 of reloading than in all other groups. Similarly, phosphorylated S6 ribosomal protein levels were significantly higher in HLU animals subjected to 3 days of reloading than in all other groups.

CaN levels tended to be greater in the soleus muscle of all HLU groups than in Con but did not reach significance until 10 days of reloading (Fig. 4).

Separation of soleus muscle MHC isoforms by gel electrophoresis is shown in Fig. 5A. MHC isoform composition in the soleus expressed as a percentage of the total MHC is shown in Fig. 5B. Soleus muscles in Con groups consisted of only MHC I (74.7–83.8%) and IIa (25.3–16.2%). In contrast, in all HLU groups, MHC IId/x was contained in the soleus of each experimental group (4.9±0.7%, 6.1±1.0%, and 2.3±0.5% at 0, 3, and 10 days, respectively). MHC IId/x content decreased in the soleus muscles of HLU animals as a function of reloading time. Finally, the MHC IIb isoform was detected in only one of the eight HLU animals at 0 and 3 days of recovery.
Despite the decrease in cell number, the exercise-trained muscle increased in size and weight in proportion to the body weight of young Wistar rats used in the present study. In contrast, growing evidence reveals that CaN plays a prominent role in muscle hypertrophy induced by IGF-I (29). Moreover, administration of rapamycin to regenerating rat soleus muscles can prevent the increase in muscle fiber size normally induced by activated PKB/Akt (27).

In the present study, we found that the phosphorylation of PKB/Akt and p70S6K increased in the soleus muscle during the early recovery period (i.e., day 3 of muscle reloading). Phosphorylated ribosomal protein S6 levels also increased at day 3 of recovery from HLU, and this elevated level persisted at day 10 of muscle reloading. Collectively, all the aforementioned results implicate PKB/Akt and its downstream mediators in the facilitation of muscle protein synthesis during recovery from muscle atrophy.

Role of CaN during recovery from atrophy. The Ca^{2+}/calmodulin-dependent phosphatase CaN has emerged as a possible candidate in the signaling of skeletal muscle fiber growth and phenotype changes (4, 10, 12–15, 19, 21, 22, 26, 30, 32). Furthermore, it has been shown that CaN signaling contributes to the initial events of myogenic differentiation through an NFATc3-dependent mechanism (10). However, the...
role of CaN in muscular hypertrophy remains controversial, inasmuch as one report indicates that mechanical overloading-induced fiber hypertrophy can be suppressed by inhibition of CaN activity via CsA (12) and another report suggests that CsA treatment does not result in muscle hypertrophy (5). Moreover, injection of CsA in normal rats does not alter fiber cross-sectional area of the soleus muscle (4, 14, 19). Therefore, although numerous findings suggest that CaN is an important signaling molecule in skeletal muscle, additional research is required to delineate the precise role of CaN in the regulation of muscle growth.

Regarding the participation of CaN in the regrowth of atrophied soleus muscle, Mitchell et al. (19, 20) suggested that CaN and muscle precursor cells are required for soleus muscle regrowth from atrophy at later stages of recovery but that CaN is not involved during early stages of recovery from atrophy. In agreement with these findings, the present data reveal that CaN expression in the atrophied soleus muscle was not elevated rapidly after muscle reloading. However, we observed that muscle CaN levels were elevated at day 10 of recovery. This finding agrees with the work of Mitchell et al. (19) and is consistent with our hypothesis that CaN plays a role in muscle remodeling from muscle atrophy in the later stages of recovery. However, in contrast to our results, Childs et al. (8) showed that CaN levels were elevated at 3 days of recovery from muscle atrophy. The reason for this discrepancy is not clear. Nonetheless, on the basis of the present data and the work of Childs et al., it seems possible that the peak CaN level in muscle may appear between day 3 and day 10 of recovery from muscle atrophy.

Therefore, judging from the changes in muscle levels of PKB/Akt, p70S6K, and S6 pathways and CaN revealed in this study, it appears that the recovery of atrophied muscle is facilitated by increased mRNA translation during the early stages of recovery followed by changes in gene transcription and translation. In agreement with this conclusion, Childs et al. (8) suggested that the cellular signaling pathways associated with protein translation (PKB/Akt, p70S6K, and 4E-BP1) were increased early during periods of regrowth from skeletal muscle atrophy, whereas the cellular signaling pathways associated with increased gene transcription and translation (i.e., CaN) were activated during later periods of recovery.

CaN has also been shown to be important for fiber type determination in skeletal muscle (1, 9, 25, 26, 30, 33, 35). Enhancer elements responding to the CaN-regulated transcription factor NFAT have been identified in the promotors of slow fiber-specific genes (9). In this regard, Nave et al. (25) reported an increased number of slow muscle fibers in transgenic mice that express activated CaN. Moreover, Serrano et al. (33) found that treatment of animals with CsA or FK-506 blocked the expression of the slow fiber phenotype in electrically stimulated regenerating soleus muscles. Furthermore, Allen and Leinwand (1) showed that CaN increased the activation of the MHC IIa promoter. In the present study, we observed that the expression of MHC IIId/x was significantly increased, whereas MHC IIa content was lower, in the soleus muscle after 10 days of HLU. However, on reloading of the atrophied soleus muscle, there was a gradual fast-to-slow shift in MHC content during the 10 days of recovery. Collectively, our results are consistent with the notion that CaN is involved in the fiber type transition from MHC IIId/x to MHC IIa during recovery from muscle atrophy.

Summary and conclusions. This study investigated alterations in signal transduction pathways involved in muscle regrowth and MHC isoform transition during recovery from disuse muscle atrophy. Our results reveal that the downstream pathway of PKB/Akt is activated early in recovery from disuse muscle atrophy. This finding is consistent with the concept that the recovery of atrophied muscle is facilitated by increased mRNA translation during the early stages of recovery. Our data also indicate that muscle levels of CaN are not elevated during the early stages of muscle reloading but are increased during the later stages of recovery from disuse muscle atrophy. This observation indicates that CaN is not required for muscle regrowth during the early phase of recovery from muscle atrophy but is involved in the later stages of muscle remodeling during recovery. Furthermore, the similar time course of changes in muscle levels of CaN and MHC isoforms during recovery from atrophy is consistent with the view that CaN plays an active role in the control of MHC phenotype in skeletal muscle.

GRANTS

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