



Phosphorylated ERK1/2 protein levels are closely associated with the fast fiber phenotypes in rat hindlimb skeletal muscles

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Abstract

The relationship between the extracellular signal-regulated kinase 1 and 2 (ERK1/2), one of the mitogen-activated protein kinases (MAPKs), and mammalian skeletal muscle fiber phenotype is unclear. We looked at this relationship in three *in vivo* conditions in male Wistar rats. First, the levels of phosphorylated (active) ERK1/2 protein were closely associated with the fiber type composition of sedentary rat hindlimb muscles: highest in the superficial portion of the gastrocnemius (100% fast fibers), lower in the plantaris (~80% fast fibers), and lowest in the soleus (~15% fast fibers). Second, during growth, there was a gradual decrease in the percentage of fast fibers from 40% at 3 weeks to 1.5% at 65 weeks and a concomitant gradual decrease in the levels of phosphorylated ERK1/2 in the soleus muscle. Third, sciatic nerve denervation induced a significant decrease in the weight of both the soleus and plantaris, but a slow-to-fast fiber type shift and increase in phosphorylated ERK1/2 protein were observed only in the soleus. Although only a few fast and fast + slow hybrid fibers of the denervated soleus muscle reacted positively to the anti-phosphorylated ERK1/2 antibody by immuno-histochemical analysis, our results suggest that the phosphorylated form of ERK1/2 seems to be closely related to the fast fiber phenotype program. Further evidence for this relationship was provided by the observation that several slow fiber phenotype-specific proteins, *i.e.*, Hsp72, Hsp60, and PGC-1, changed in the opposite direction of the levels of phosphorylated ERK1/2 protein.

Keywords MAPK · ERK1/2 · Skeletal muscle · Phosphorylation · Fiber type composition · Denervation

Introduction

Mammalian skeletal muscles are composed of two types of fibers, *i.e.*, slow-twitch type I and fast-twitch type II fibers. These fiber phenotypes can be identified by their slow and fast myosin heavy chain (MyHC) isoforms, respectively, and have a high plasticity to increased or decreased levels of contractile

activity as reflected in adaptations in muscle weight/fiber size, mechanical properties, fiber type composition, MyHC profiles, and gene and protein expressions [1, 23–25].

Several studies have reported that the mitogen-activated protein kinase (MAPK) signaling pathway has an important role in maintaining skeletal muscle weight [29, 30] and in the proliferation and differentiation of myofibers [2, 12, 32]. In addition, several types of muscle activity, such as resistance, interval, and/or sprint training, activate this signaling pathway, resulting in muscle fiber adaptations [3, 13, 31]. At least four MAPK subfamilies have been identified, which are as follows: extracellular signal-regulated kinase 1 and 2 (ERK1/2), c-Jun N-terminal kinase (JNK), p38, and ERK5 [9, 22]. The ERK1/2 subfamily has a molecular weight of 42 and 44 kDa, respectively, and *in vitro* experiments using C2C12 or MM14 cells suggest that ERK1/2 and/or ERK2 are necessary for differentiation, expression of MyHC isoforms, and formation of multinucleated myofibers [6, 10].

The role of ERK1/2 in determining the phenotype of a muscle fiber is unclear. Some studies have reported that ERK1/2 is closely related to the expression of the slow type I fiber

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phenotype [4, 5, 14, 15]. For example, Meissner et al. [14] reported that in vitro and in vivo electrical stimulation activated ERK1/2 phosphorylated transcriptional co-activator p300 that, in turn, led to the acetylation of NFAT and enhanced NFAT–DNA binding, resulting in the up-regulation of slow type MyHC I gene expression. In addition, inhibition of calcineurin or the ERK1/2 pathway reduced the slow MyHC I mRNA and up-regulated the fast MyHC IIx and IIb isoforms in skeletal muscle myotubes, suggesting the calcineurin and ERK1/2 pathways up-regulate slow MyHC I and suppress fast MyHC isoforms [5].

In contrast, other studies suggest a strong effect of ERK1/2 in up-regulating the fast type II fiber phenotype [27, 28]. For example, Shi et al. [28] reported that overexpression of constitutively active ERK2 in mouse and rat hindlimb muscles resulted in an enhancement of the fast type MyHC IIb isoform and a depression of slow MyHC I, while pharmacological blocking of ERK1/2 signaling increased the levels of slow MyHC I. Moreover, activation of ERK2 signaling induced up-regulation of soleus fast MyHC IIb reporter gene but not the slow MyHC I. These authors concluded that ERK1/2 pathway was necessary to preserve the fast-twitch fiber phenotype along with a concomitant repression of the slow-twitch fiber phenotype. Thus, the effects of the active (phosphorylated) form of ERK1/2 signaling pathway on fiber type specificity are still unclear.

The primary purpose of the present study, therefore, was to clarify the relationship between ERK1/2 proteins and the fiber type composition of rat hindlimb skeletal muscles. This relationship was determined in (1) muscles having different fiber type compositions in adult control rats, (2) the soleus muscle in rats ranging in age from 3 to 65 weeks as the muscle fiber type composition was progressing towards a higher percentage of slow fibers, and (3) the soleus after denervation when the muscle fiber type composition was progressing towards a higher percentage of fast fibers. In addition, the levels of other proteins related to fiber type specificity were determined, i.e., heat-shock protein (Hsp) 72, a heat stress-inducible molecular chaperone, and mitochondrial Hsp60 that are known to be closely related to the slow type I fiber phenotype [16, 19] and the transcriptional co-activator PGC-1 that is known to stimulate mitochondrial biogenesis and oxidative enzymes and thus also related to the expression of slow type I fiber phenotype [11, 26]. The results of the present study strongly suggest that the levels of phosphorylated ERK1/2 proteins are closely related to the fast phenotype composition of rat hindlimb muscles.

Materials and methods

Animals and experimental procedures

All experimental and animal care procedures were conducted in accordance with the Japanese Physiological Society *Guide for the Care and Use of Laboratory Animals*. This study was

approved by the Animal Use Committee at Kumamoto University.

Three separate experiments were performed.

Experiment 1: The soleus (Sol), plantaris (Pla), and medial gastrocnemius (Gas) muscles of adult 10-week-old male Wistar rats ($n = 8$, mean \pm SE body weight = 358 ± 6.1 g) were excised bilaterally, wet weighed, and analyzed using immuno-histochemical and biochemical procedures. The mean Sol, Pla, and Gas weights were 143 ± 6.0 , 380 ± 10.8 , and 784 ± 16.0 mg, respectively.

Experiment 2: The Sol muscles of 3-, 10-, 20-, 46-, and 65-week-old male Wistar rats were excised, wet weighed, and analyzed using immuno-histochemical and biochemical procedures. The number of rats used was 8 at each week of age, except for the 65-week time point ($n = 6$).

Experiment 3: The Sol and Pla muscles from 10-week-old sedentary control (Con, $n = 8$) and denervated (Den, $n = 8$) male Wistar rats were excised bilaterally, weighed, and analyzed using immuno-histochemical and biochemical procedures. Denervation involved removing a 10-mm segment of the sciatic nerve bilaterally at the 9-week time point and maintaining the rats for 1-week post-injury.

Immuno-histochemical analyses

The muscles from the left side were frozen in isopentane, cooled with liquid nitrogen, and used for the immuno-histochemical analyses. Serial cross-sections (10- μ m thick) from the mid-portion of the muscle were cut at -20 °C using a cryostat. The sections were air-dried and fixed with 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS, pH 7.2) for 15 min. The sections, then, were treated with 100% methanol at -20 °C for 10 min and further incubated in 0.1 M PBS with 10% normal serum and 1% Triton X-100 for 60 min at room temperature to block non-specific staining. For fiber type classification, the sections were reacted overnight at 4 °C with the primary anti-fast myosin (M4276, Sigma, Saint Louis, MO, USA) and anti-slow myosin (M8421, Sigma) antibodies diluted 1:100 with 0.1 M PBS with 5% normal goat serum and 0.3% Triton X-100. The sections were washed twice for 10 min each in 0.1 M PBS and incubated with the secondary Alexa Fluor 488 goat anti-mouse IgG (A-11001, Invitrogen Japan, Tokyo, Japan) and Alexa Fluor 546 goat anti-rabbit IgG (A-11010, Invitrogen Japan, Tokyo, Japan) diluted 1:200 with 0.1 M PBS with 5% normal serum and 0.1% Triton X-100 for 60 min at room temperature. To identify the basement membrane, the sections were reacted with primary anti-laminin antibody (1:200, L9393, Sigma). The sections, then, were washed, air-dried, and cover-slipped.

Based on the immuno-histochemical reactivity, individual fibers were classified as slow (positive only to the anti-slow antibody), fast (positive only to the anti-fast antibody), or

slow/fast hybrid (positive to both antibodies). Between 1000 and 2000 fibers were analyzed in the cross-section of each muscle/muscle portion in each experiment to determine the muscle fiber type composition. In experiment 1, both a superficial (GasS, away from the bone) and a deep (GasD, close to the bone) portion of the Gas were analyzed. In experiment 3, immuno-histochemical reactivity to the primary antibody of phosphorylated ERK1/2 (4370S, Cell Signaling Japan, Tokyo, Japan) was used to clarify the fiber type specificity using the same procedures described previously.

Muscle sample preparation for biochemical protein analyses

The muscles from the right side were used for the biochemical analyses. The Gas was separated into a superficial (GasS, white appearance) and a deep (GasD, red appearance) portion. Each muscle/muscle portion in each experiment was homogenized with 10 volumes of buffer containing 10 mM Tris/HCl (pH 7.6), 10 mM NaCl, 0.1 mM EDTA, and 15 mM mercaptoethanol, and then centrifuged at 12,000 *g* for 20 min. The supernatants were boiled in a sample buffer for 2 min, adjusted to a final protein concentration at 1 $\mu\text{g}/\mu\text{l}$, and then subjected to sodium-dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting.

SDS-PAGE and Western blotting

A total of 20 μg of protein was applied and separated by SDS-PAGE. Electrophoresis was continued at 30 mA (constant current/gel) for ~ 50 min, and the gel was subjected immediately to Western blotting. Proteins on the gel were transferred to PVDF membranes (IPVH00010, Immobilon-P, Merck Millipore, Germany) using a semi-dry blotting unit (Fisher Biotech FB-SDB-2020, Fisher Scientific Japan, Tokyo, Japan) at 50 mA (constant current/gel) for 2–3 h, and blocked with 5% non-fat milk in Tris-buffered saline (TBS; 100 mM Tris-HCl, pH 7.5, 0.9% NaCl) with 0.1% Tween 20 (TTBS) for 1 h at room temperature. After being washed twice in TTBS for 15 min each, the blots were incubated with primary Hsp72 (SPA-810, StressGen, BC, Canada), Hsp60 (SPA-806, StressGen), PGC-1 (AB3242, Chemicon, CA, USA), and total or phosphorylated ERK1/2 (9100S p44/42 MAPK Ab Kit, Cell Signaling Japan, Tokyo, Japan) antibodies, diluted 1:500–2000 in TTBS, overnight at 4 °C. The membranes were washed twice for 15 min each in TTBS and then reacted with horseradish peroxidase-conjugated anti-mouse IgG (A-9044 diluted 1:1000 in TTBS, Sigma) or anti-rabbit IgG (A-6145 diluted 1:1000 in TTBS, Sigma) for 2 h at room temperature. For detection of Hsp72, Hsp60, and PGC-1 proteins, the membranes were washed twice in TTBS and once in TBS for 10 min each and reacted for ~ 10 min with H_2O_2 solution

(diluted 1:1000 with TBS) using 3,3-diaminobenzidine (D5637, Sigma) as a substrate. The total and phosphorylated ERK1/2 proteins were detected by enhanced chemiluminescence. Quantification of detected protein was performed using a laser scanning densitometer (densitograph 4.0, Mac AE-6920 MF, ATTO). Both the 42 and 44 kDa bands were quantified for the ERK 1/2 proteins, and monoclonal anti-actin antibody (A-4700 diluted 1:1000 in TTBS, Sigma) was used as a loading control.

Statistical analyses

All data are presented as means \pm SE. Significant differences between muscles in experiment 1 or week of age in experiment 2 were determined using one-way ANOVA followed by Fisher's post-hoc test. Significant differences between groups in experiment 3 were determined using unpaired Student's *t* tests. Statistical significance was established at $p < 0.01$. Pearson's correlation coefficient was measured by using statistical software StatView-J 5.0 between the mean values of the levels of phosphorylated ERK1/2 protein and the percentage of fast fibers in experiments 1 and 2 or the percentage of slow/fast hybrid fibers in experiment 2.

Results

Fiber type composition in 10-week-old sedentary rat hindlimb muscles (experiment 1)

Figure 1a shows the representative immuno-histochemical fiber type staining patterns for the Sol, Pla, GasD, and GasS. Figure 1b indicates the percentage of slow, fast, and slow/fast hybrid fibers in each muscle/muscle portion. The Sol had a significantly higher percentage of slow fibers ($\sim 80\%$) and a significantly lower percentage of fast fibers ($\sim 14\%$) than all the other muscle/muscle portions. The GasS was comprised only of fast fibers. The order of the percentage of slow fibers was Sol > GasD > Pla > GasS, and that for the fast fibers was GasS > Pla > GasD > Sol. The percentage of hybrid fibers was relatively low, i.e., 0% in the GasS and between 6 and 8% in the other muscle/muscle portions.

Levels of Hsp72, Hsp60, PGC-1, and ERK1/2 proteins in 10-week-old sedentary rat hindlimb muscles (experiment 1)

All protein levels are expressed relative to the Sol values (Fig. 2, Sol values = 100%). The levels of Hsp72 (Fig. 2a) and Hsp60 (Fig. 2b) were significantly different among all four muscle/muscle portions. The order of the values reflected the percentage of slow fibers (see Fig. 1b), i.e., Sol > GasD >

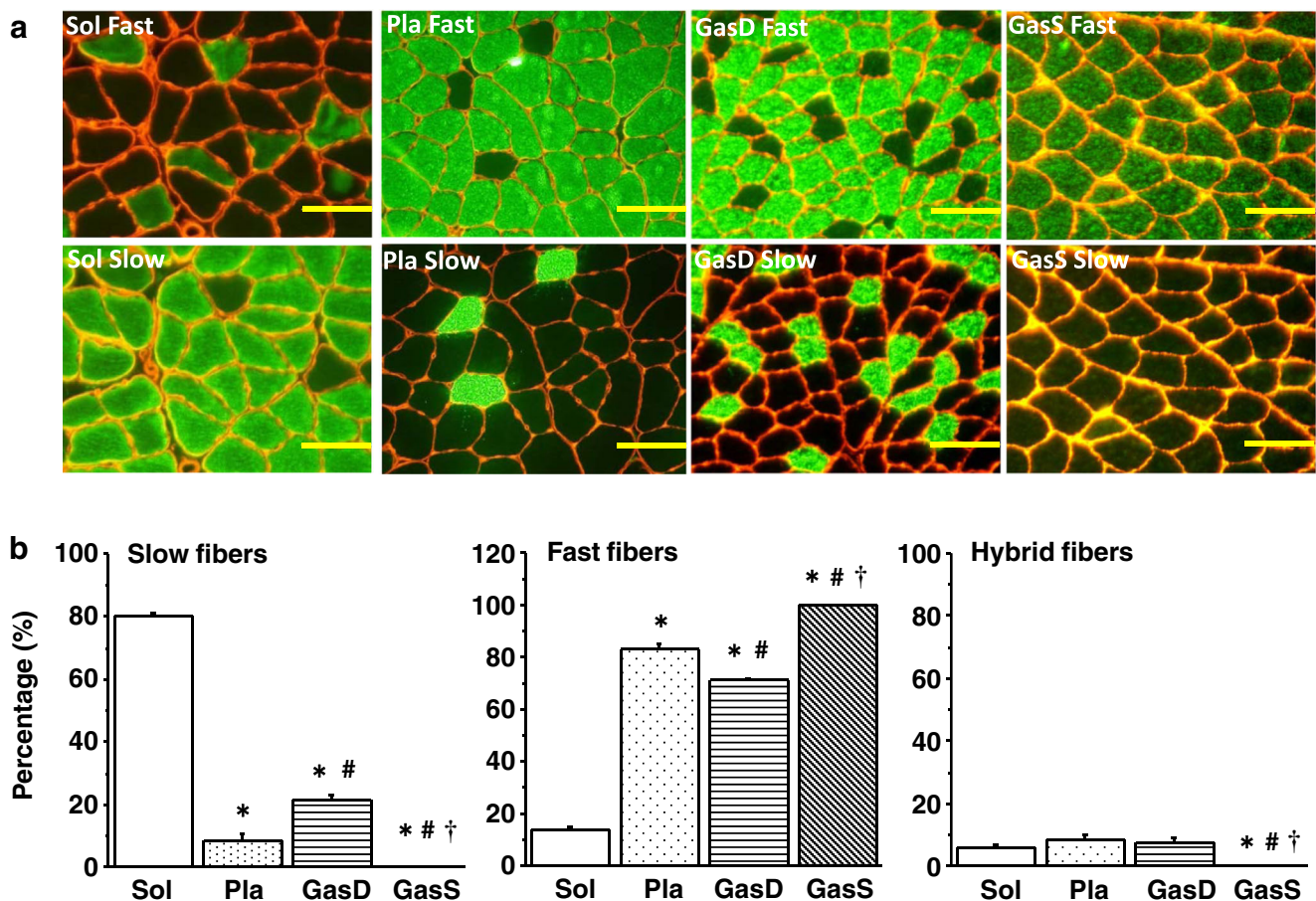


Fig. 1 Representative immuno-histochemical fast (upper panels) and slow (lower panels) fiber staining patterns (**a**) and fiber type composition (**b**) of the soleus (Sol), plantaris (Pla), and the deep (GasD) and superficial (GasS) portions of the gastrocnemius muscles of 10-week-old sedentary

rats (experiment 1, $n = 8$). Fibers were classified as slow, fast, or slow/fast hybrid. Scale bars in (**a**) = 100 μm . Values in (**b**) are mean \pm SEM. *, #, and †, significantly different from the Sol, Pla, and GasD, respectively, at $P < 0.01$

Pla > GasS. The levels of PGC-1 were similar in the Sol and GasD, lower in the Pla and GasS than the Sol, and lower in the GasS than the GasD, i.e., showing a similar rank order as the heat-shock proteins (Fig. 2a–c). No differences in total ERK1/2 were observed among muscles/muscle portions (Fig. 2d), whereas the levels of phosphorylated ERK1/2 protein were higher in the GasS, Pla, and GasD than in the Sol, higher in the Pla than in the GasD, and higher in the GasS than in the GasD (Fig. 2e). There was a strong correlation ($r = 0.889$) between the mean values of the percentage of fast fibers and the levels of phosphorylated ERK1/2 protein.

Body and Sol weights in growing and aging rats (experiment 2)

Body weight (Fig. 3a) and absolute Sol weight (Fig. 3b) were significantly larger compared to the previous age at all other time points, except for a smaller Sol weight at 65 compared to 46 weeks of age ($p > 0.01$). Relative Sol weight to body weight was not different at 3 and 10 weeks and thereafter

was significantly smaller with the values at 65 weeks being lower than at all the other time points (Fig. 3c).

Fiber type composition of the Sol in growing and aging rats (experiment 2)

Figure 4a shows representative immuno-histochemical fiber type staining patterns for the Sol at the 3-, 20-, and 65-week time points. The percentage of slow fibers was lowest at 3 weeks of age and progressively increased thereafter with the values being not different at 46 and 65 weeks of age (Fig. 4b). In contrast, the percentage of fast and slow/fast hybrid fibers was higher at 3 weeks than at all the other time points, except that the percentage of hybrid fibers was similar at the 3- and 10-week time points and gradually decreased with growth.

Levels of Hsp72, Hsp60, PGC-1, and ERK1/2 proteins in the Sol of growing and aging rats (experiment 2)

Figure 5a shows the representative expression pattern of each protein examined from 3 to 65 weeks of age: the protein level

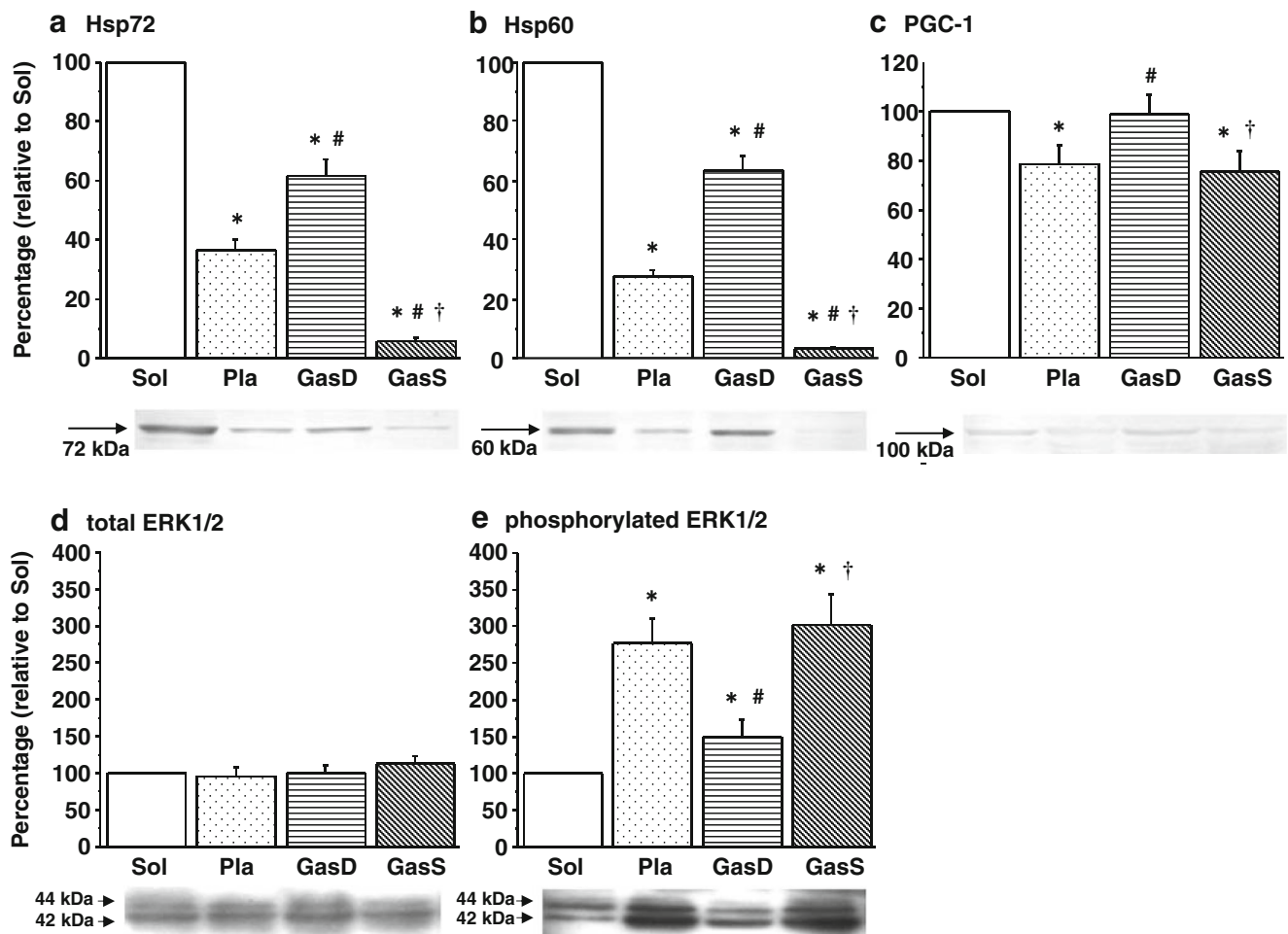


Fig. 2 Levels of Hsp72 (a), Hsp60 (b), PGC-1 (c), and total (d) and phosphorylated (e) ERK1/2 proteins in the Sol, Pla, GasD, and GasS muscles of 10-week-old sedentary rats (experiment 1). Values are mean

± SEM and expressed relative to the Sol level (100%). *, #, and †, significantly different from the Sol, Pla, and GasD, respectively, at $P < 0.01$

is normalized to actin protein that was used as a loading control. The levels of Hsp72 (Fig. 5b), Hsp60 (Fig. 5c), and PGC-1 (Fig. 5d) proteins were increased significantly with

increasing age. The levels of Hsp72 were higher at 46 and 65 weeks than at 3 weeks and higher at 46 weeks than at 10 weeks. The levels of Hsp60 were higher at 20, 46, and

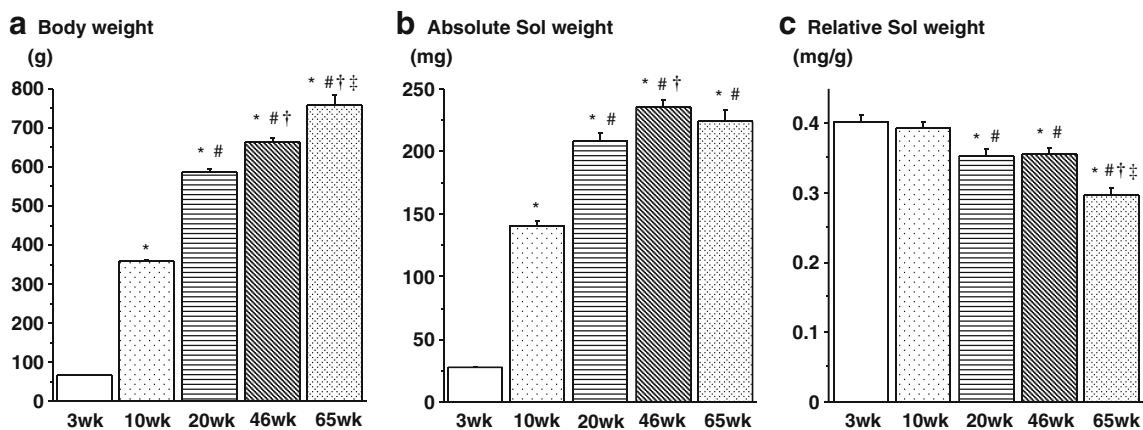


Fig. 3 Body weight (a) and absolute (b) and relative (to body weight, c) Sol weights of 3- to 65-week-old rats (experiment 2, $n = 8$ at the 3–46-week time points and $n = 6$ at the 65-week time point). Values are mean ±

SEM. *, #, †, and ‡, significantly different from 3, 10, 20, and 46 weeks, respectively, at $P < 0.01$

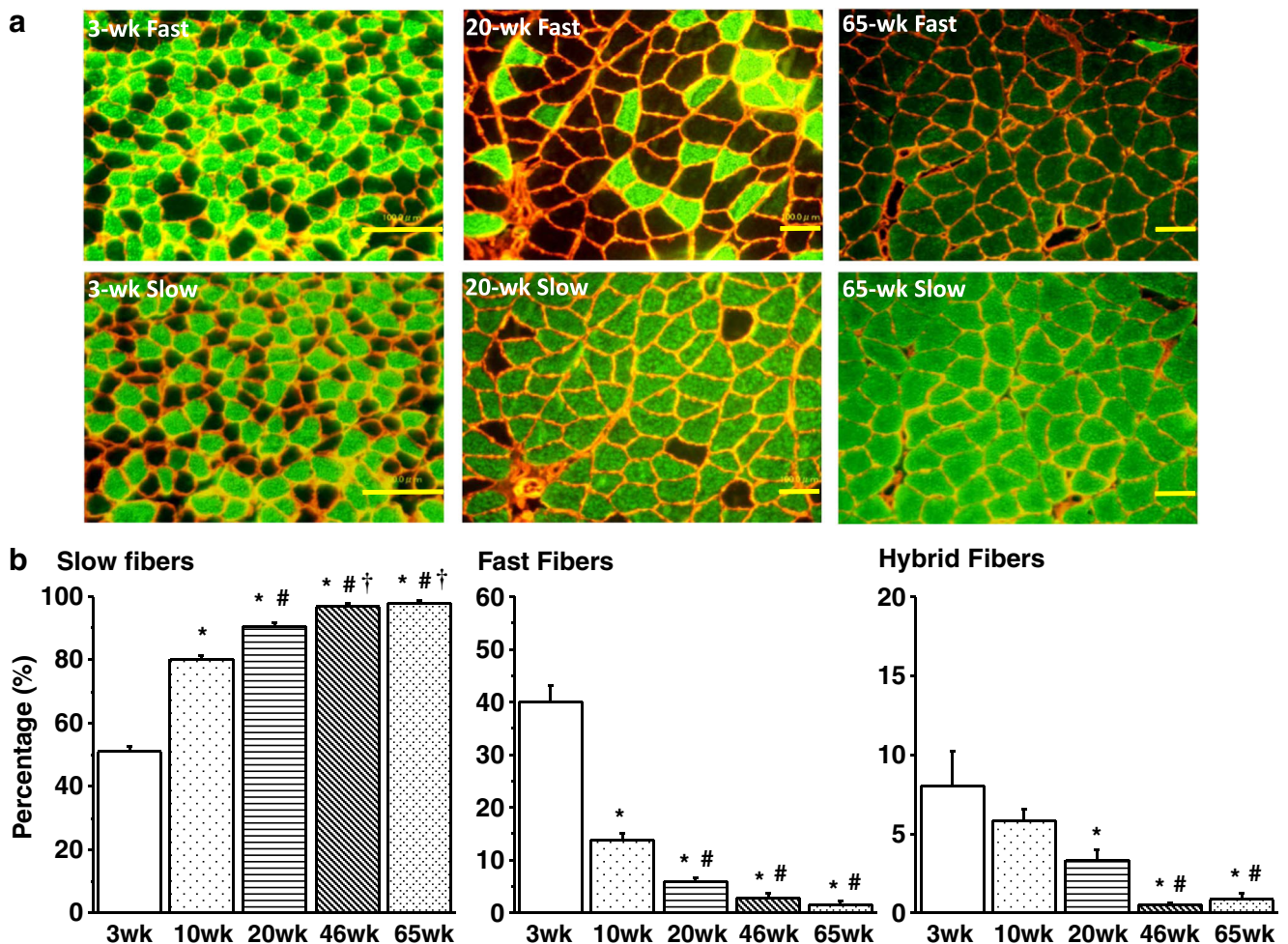


Fig. 4 Representative immuno-histochemical fast (upper panels) and slow (lower panels) fiber staining patterns of the Sol of 3-, 20-, and 65-week-old rats (**a**) and fiber type composition (**b**) from 3- to 65-week-old rat Sol (experiment 2). Fibers were classified as slow, fast, or slow/fast

hybrid. Scale bars in (**a**) = 100 μ m. Values in (**b**) are mean \pm SEM. *, #, and †, significantly different from 3, 10, and 20 weeks, respectively, at $P < 0.01$

65 weeks than at 3 weeks and higher at 46 and 65 weeks than at 10 weeks. The levels of PGC-1 were higher at 46 and 65 weeks than at 3 and 10 weeks. These patterns of change over time were similar to those observed for the increase in the percentage of slow fibers over time (see Fig. 4b). Total ERK1/2 levels remained constant throughout the experimental period (Fig. 5e). In contrast, the levels of phosphorylated ERK1/2 protein progressively decreased over time and were lower at 20, 46, and 65 weeks compared to those at 3 weeks (Fig. 5f). In addition, the level of phosphorylated ERK1/2 protein at the 65-week time point was almost non-existent ($\sim 1\%$) and was lower than at all the other time points. This pattern of change over time was similar to that observed for the decrease in the percentage of fast and slow/fast hybrid fibers over time (see Fig. 4b). There was a strong correlation between the mean values of the levels of phosphorylated ERK1/2 protein and the percentage of fast fibers ($r = 0.940$) or slow/fast hybrid fibers ($r = 0.901$).

Body and Sol and Pla weights of Con and Den rats (experiment 3)

The body (Fig. 6a), absolute Sol (Fig. 6b) and Pla (Fig. 6c), and relative (vs. body weight) Sol (Fig. 6d) and Pla (Fig. 6e) weights were smaller for the Den compared to the Con group.

Fiber type composition of the Sol and Pla muscles of Con and Den rats (experiment 3)

Figure 7a shows representative immuno-histochemical fiber type staining patterns for the Sol and Pla of Con and Den rats. The percentage of slow fibers was lower and that of slow/fast hybrid fibers higher in the Sol of Den than Con rats (Fig. 7b). The fiber type composition for the Pla was not different between groups (Fig. 7b).

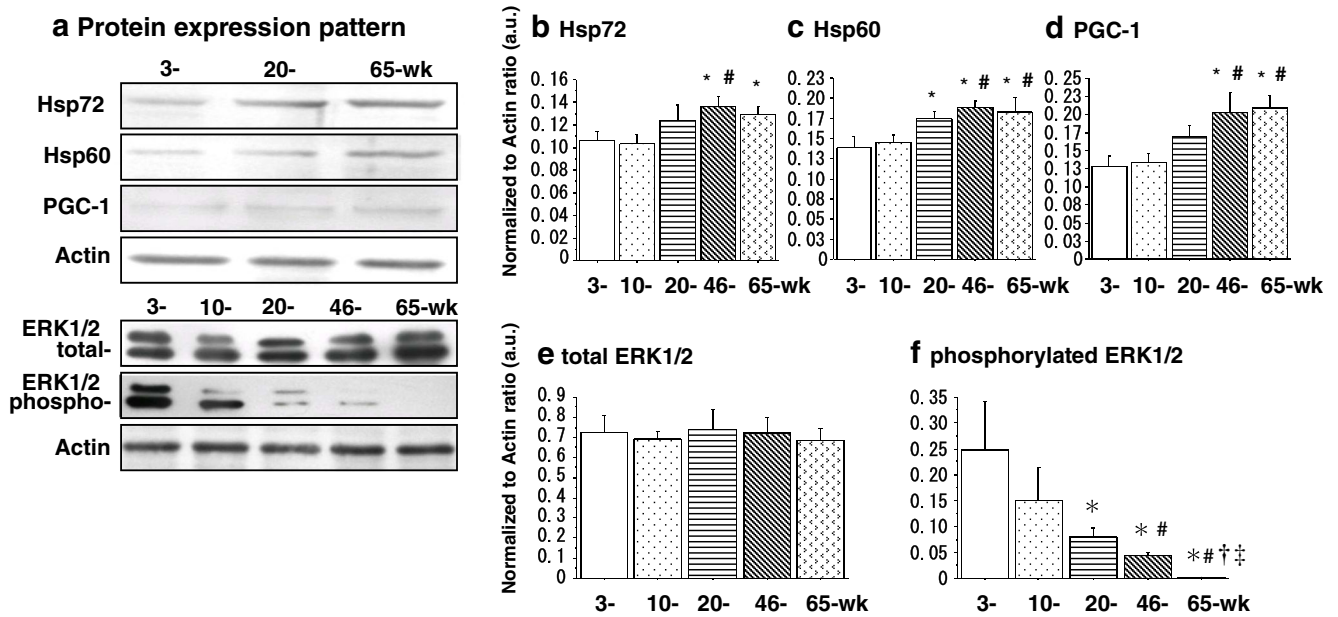


Fig. 5 Representative protein expression pattern (a) and the levels of Hsp72 (b), Hsp60 (c), PGC-1 (d), and total (e), and phosphorylated (f) ERK1/2 proteins in the Sol of 3- to 65-week-old rats (experiment 2).

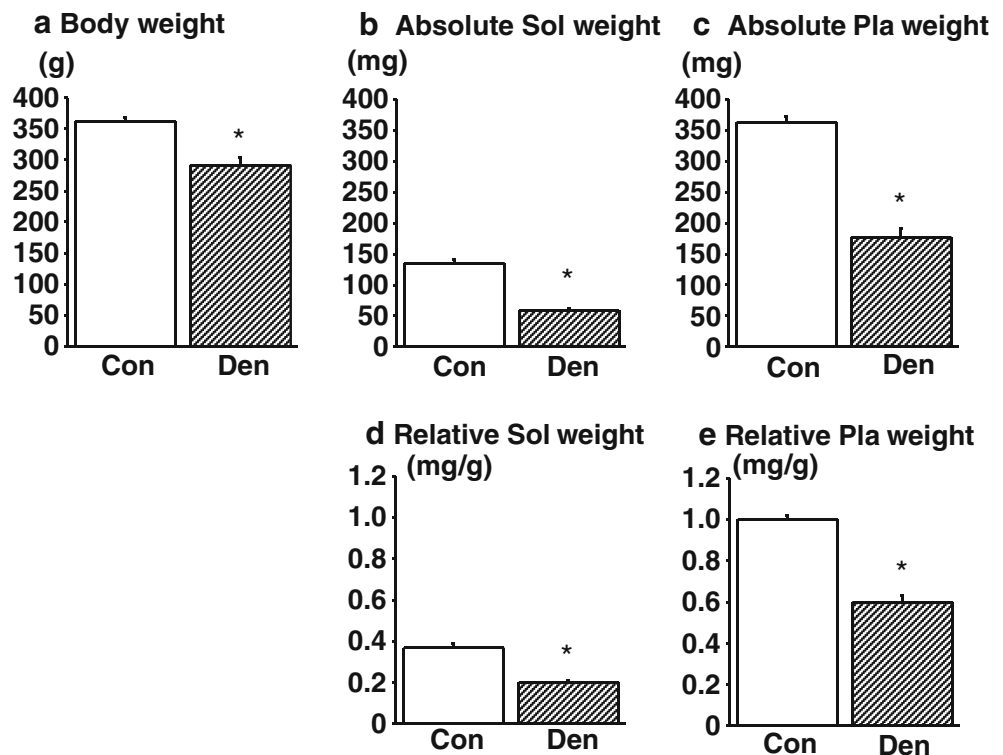
Values are mean ± SEM and normalized to the levels of actin protein that was used as a loading control. *, #, †, and ‡, significantly different from 3, 10, 20, and 46 weeks, respectively, at $P < 0.01$. a.u., arbitrary unit

Levels of Hsp72, Hsp60, PGC-1, and ERK1/2 proteins in the Sol and Pla of Con and Den rats (experiment 3)

Figure 8a shows representative expression patterns of each protein examined in the Sol and Pla of Con and Den groups:

the protein level is normalized to actin protein that was used as a loading control. The levels of Hsp72, Hsp60, and PGC-1 protein in the Sol were lower in the Den than in the Con group (Fig. 8b). The levels of total ERK1/2 in the Sol were not different between groups, whereas the levels of

Fig. 6 Body weight (a) and absolute (b and c) and relative (to body weight, d and e) weights of the Sol and Pla of 10-week-old control (Con, $n = 8$) and dener- vated (Den, $n = 8$) rats (experi- ment 3). Values are mean ± SEM. *, significantly different from Con at $P < 0.01$



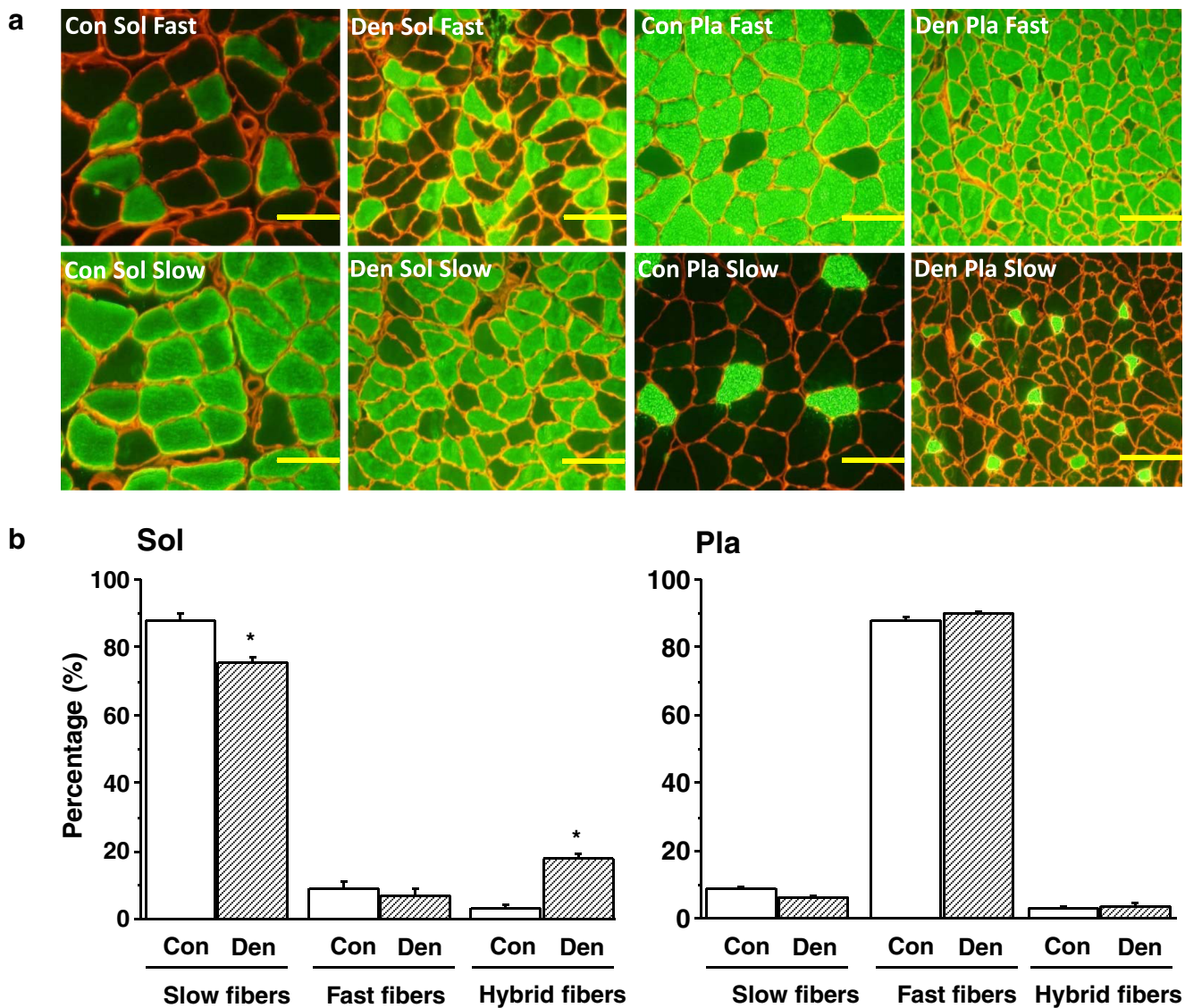


Fig. 7 Representative immuno-histochemical fast (upper panels) and slow (lower panels) fiber staining patterns (**a**) and fiber type composition (**b**) of the Sol and Pla muscles of Con and Den rats (experiment 3). Fibers

were classified as slow, fast, or slow/fast hybrid. Scale bars in (**a**) = 100 μ m. Values in (**b**) are mean \pm SEM. *, significantly different from Con at $P < 0.01$

phosphorylated ERK1/2 in the Sol were higher in the Den than in the Con group (Fig. 8b). There were no differences between groups for any of the protein levels in the Pla (Fig. 8c).

Fiber type specificity of phosphorylated ERK1/2 protein in the Sol and Pla of Con and Den rats based on immuno-histochemical analyses (experiment 3)

Immuno-histochemical staining was performed to examine directly the fast fiber specificity of phosphorylated ERK1/2 protein in the Sol and Pla of Con and Den rats (experiment 3). No specific staining of phosphorylated ERK1/2 protein was observed in all fiber types of the Con Sol, Con Pla, or Den Pla, as shown in Fig. 9a–f, while only a few fast fibers (arrows # 1–

3 in Fig. 9g–i) or fast + slow hybrid fibers (arrow # 4 in Fig. 9g–i) in the Den Sol were stained positively to the anti-phosphorylated ERK1/2 antibody, in which the percentage of those positive fibers was very low, i.e., ranged 1–5%.

Discussion

The novelty of the present study is that we used three in vivo approaches to determine the relationship between the fiber phenotype and levels of phosphorylated ERK1/2 in rat hindlimb muscles. The primary findings of the present study show that the levels of phosphorylated ERK1/2 protein are associated closely with the percentage of fast fibers in the muscles of sedentary rats, the decrease in the percentage of

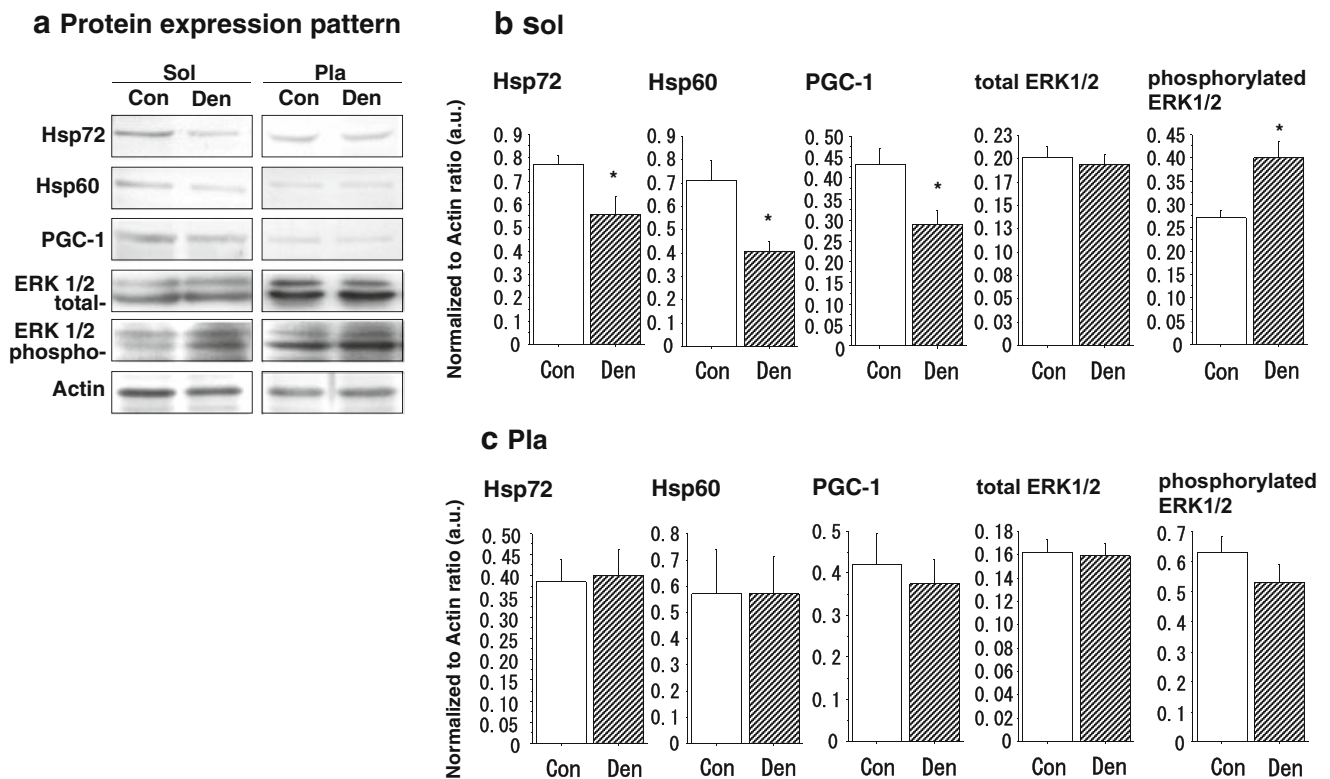


Fig. 8 Representative protein expression patterns (a) and the levels of Hsp72, Hsp60, PGC-1, and total and phosphorylated ERK1/2 proteins of the Sol (b) and Pla (c) in Con and Den rats (experiment 3). Values are

mean \pm SEM and normalized to the levels of actin protein that was used as a loading control. *, significantly different from Con at $P < 0.01$. a.u., arbitrary unit

fast fibers in the Sol during growth, and the increase in the percentage of fast fibers in the Sol associated with denervation. In addition, the levels of Hsp72, Hsp60, and PGC-1 were associated closely with the percentage of slow fibers in the same experimental models, findings consistent with previous studies [11, 16, 19, 26].

In experiment 1, the levels of phosphorylated ERK1/2 protein were 2.7–3.0-fold higher in the predominantly fast Pla and GasS than in the predominantly slow Sol in sedentary rats, whereas the total ERK1/2 was not different among the muscle/muscle portions (Fig. 2). Shi et al. [28] reported similar results, i.e., the levels of phosphorylated ERK 2 protein were 2.3–2.5-fold higher in the predominantly fast extensor digitorum longus than the Sol muscle of sedentary rats and mice. These authors also reported a significant decrease in the levels of fast type MyHC IIb and sarcoplasmic reticulum calcium ATPase (SERCA) 1 in C2C12 cells when ERK1/2 signaling was blocked by the MEK (MAPK kinase) inhibitor PD98059, while over-expression of active ERK2 resulted in an up-regulation of these proteins in both in vitro and in vivo experiments. The authors concluded that ERK1/2 signaling was necessary to preserve the fast fiber phenotype and repress the slow fiber phenotype. The present results are in concert with this conclusion.

In experiments 2 and 3, we show that the levels of phosphorylated ERK1/2 protein, but not total ERK1/2, are closely

associated with changes in the fiber type composition of the Sol muscle, i.e., a decrease with a decrease in the fast fiber composition with growth (Figs. 4 and 5E) and an increase with an increase in the fast fiber composition following denervation (Figs. 7 and 8C). Similar results have been reported using other models of muscle plasticity. For example, administration of the beta 2-agonist clenbuterol induces muscle fiber hypertrophy and a slow-to-fast fiber phenotype shift with de novo expression of fast MyHC IIx and IIb isoforms in rodent hindlimb muscle fibers [18, 20, 27]. These adaptations were blunted and/or blocked by treatment with a MEK1/2–ERK1/2 signaling inhibitor [27], reflecting the importance of this signaling pathway for these adaptations. Hindlimb unloading results in fiber atrophy and a slow-to-fast fiber phenotype shift, particularly in the Sol [17, 21], and reloading the hindlimbs restores fiber size and the slow fiber phenotype in the Sol [7]. Up-regulation of phosphorylated ERK1/2 was reported in the Sol of 5–10-day hindlimb unloaded rats and then was returned to near-normal levels after 10 days of reloading [8]. Combined, these data strongly suggest that the active (phosphorylated) form of ERK1/2 is related to the fast fiber phenotype expression. The precise mechanism(s) involved with this relationship are unclear and remain to be elucidated.

Unfortunately, only a few fast and fast + slow hybrid fibers of the Den Sol reacted positive staining to anti-phosphorylated

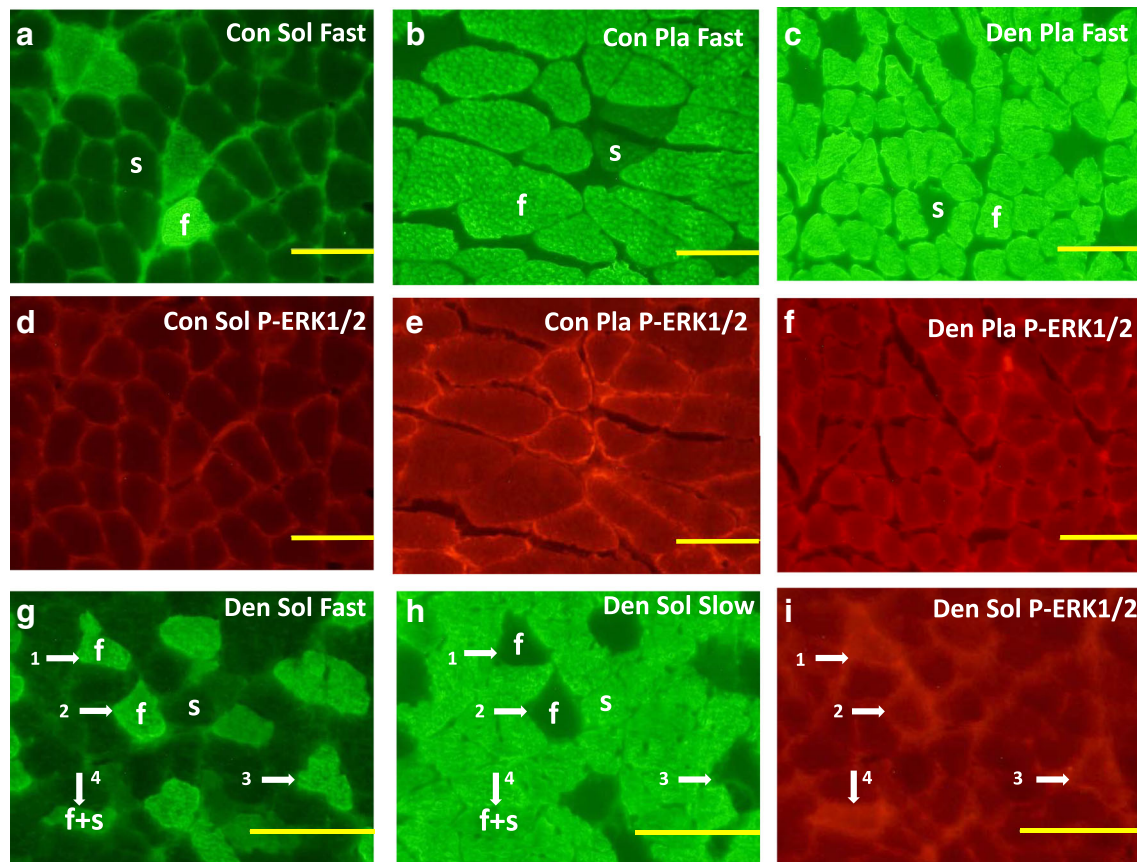


Fig. 9 Representative immuno-histochemical staining patterns showing fast fibers (panels a–c and g), slow fibers (panel h), and phosphorylated ERK1/2 protein (panels d–f and i) for the Sol and Pla in Con and Den rats (experiment 3). Fibers were classified as fast, slow, or fast/slow hybrid.

Scale bars in all panels = 100 μ m; note that there is a larger magnification in panels g–i than for the other panels. f, fast fibers; s, slow fibers, f + s, fast/slow hybrid fibers

ERK1/2 antibody by immuno-histochemical analysis performed on the muscles of experiment 3 (Fig. 9). No studies have proved the immuno-histochemical specificity of phosphorylated ERK1/2 protein to muscle fiber type, and the reason(s) why only those fibers indicated positive reaction in the present study are unclear. One possible explanation may be that small amount of phosphorylated ERK1/2 protein could not be reflected on fiber type specificity in the Con Sol, Con Pla, or Den Pla by immuno-histochemical analysis. On the other hand, biochemical analysis indicated the increase of phosphorylated ERK1/2 protein in atrophied Den Sol fibers (Fig. 8); within those fibers, only a few fast and fast + slow hybrid fibers expressing higher level of phosphorylated ERK1/2 protein may be reacted positively by immuno-histochemical analysis.

In contrast to the findings discussed previously, other studies have reported that the ERK1/2 pathway is related to the slow fiber phenotype expression. For example, Dupont et al. [4] reported a 40–50% down-regulation of phosphorylated ERK1/2 protein in the rat Sol after 7–28 days of hindlimb unloading, concomitant with a depression of the slow MyHC isoform, de novo synthesis of the fast MyHC Iix and

Iib isoforms, and a \sim 2.5-fold increase in glycolytic metabolism-related enzyme activity. In addition, low-frequency electrical stimulation to the Sol, which is known to enhance the slow fiber phenotype, prevented all of these changes. Murgia et al. [15] used in vivo transfection of active Ras, which activates the MAPK (ERK1/2) pathway, in the regenerating Sol after bupivacaine injection with denervation enhanced the slow fiber phenotype program and that these effects were inhibited by a dominant-negative Ras mutant. These results suggest that the Ras-ERK1/2 pathway is important for the expression of the slow fiber phenotype in regenerating fibers.

Based on data from both Ca^{2+} ionophore-treated C2C12 myotubes and electrically stimulated mouse soleus muscles, Meissner et al. [14] reported that ERK1/2-mediated phosphorylation of transcriptional coactivator p300 was crucial for enhancing the acetylation of transcriptional function of nuclear factor of activated T cell (NFAT) c1, which is essential for Ca^{2+} -induced slow MyHC I gene expression. Higginson et al. [5] also observed that treatment with calcineurin inhibitor cyclosporine A and MEK1/2-ERK1/2 pathway inhibitor U0126 depressed slow MyHC mRNA and enhanced fast

MyHC IIX mRNA in cultured rat skeletal myotubes, suggesting both the calcineurin-NFAT and MEK1/2-ERK1/2 pathways upregulate slow MyHC gene and suppress fast MyHC expression.

In conclusion, the reasons for these conflicting results are unclear and most likely are related to the specifics of the experimental designs, i.e., in vivo vs. in vitro, animal species and strain, and protein vs. mRNA levels. The present results based on three in vivo experimental models indicate the possibility that the levels of active (phosphorylated) ERK1/2 protein are closely associated with the expression levels of fast fiber phenotypes in rat hindlimb muscles. The observation that the levels of phosphorylated ERK1/2 protein changed in the opposite direction as those of the slow fiber-specific Hsp72, Hsp60, and PGC-1 proteins provides further evidence for ERK1/2 being closely related to the fast fiber phenotype.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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