Changes in inorganic phosphate and force production in human skeletal muscle after cast immobilization

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Departments of 1Physical Therapy and 2Physiology and Functional Genomics, University of Florida, Gainesville, Florida; Departments of 3Physiology, 4Orthopedic Surgery, and 5Rehabilitation Medicine, University of Pennsylvania, Philadelphia, Pennsylvania; and 6Department of Orthopedics and Rehabilitation, University of Florida, Gainesville, Florida

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Pathare, Neeti, Glenn A. Walter, Jennifer E. Stevens, Zhaohui Yang, Enyi Okerke, John D. Gibbs, John L. Esterhai, Mark T. Scarborough, C. Parker Gibbs, H. Lee Sweeney, and Krista Vandenborne. Changes in inorganic phosphate and force production in human skeletal muscle after cast immobilization. J. Appl. Physiol. 98: 307–314, 2005. First published August 27, 2004; doi:10.1152/japplphysiol.00612.2004.—Cast immobilization is associated with decreases in muscle contractile area, specific force, and functional ability. The pathophysiological processes underlying the loss of specific force production as well as the role of metabolic alterations are not well understood. The aim of this study was to quantify changes in the resting energy-rich phosphate content and specific force production after immobilization. 31P-magnetic resonance spectroscopy, three-dimensional magnetic resonance imaging, and isometric strength testing were performed in healthy subjects and patients with an ankle fracture after 7 wk of immobilization and during rehabilitation. Muscle biopsies were obtained in a subset of patients. After immobilization, there was a significant decrease in the specific plantar flexor torque and a significant increase in the inorganic phosphate (Pi) concentration (P < 0.001) and the Pi-to-phosphocreatine (PCr) ratio (P < 0.001). No significant change in the PCr content or basal pH was noted. During rehabilitation, both the Pi content and the Pi-to-PCr ratio decreased and specific force increased, approaching control values after 10 wk of rehabilitation. Regression analysis showed an inverse relationship between the in vivo Pi concentration and specific torque (r = 0.65, P < 0.01). In vitro force mechanics performed on skinned human muscle fibers demonstrated that varying the Pi levels within the ranges observed across individuals in vivo (4–10 mM) changed force production by ~16%. In summary, our findings clearly depict a change in the resting energy-rich phosphate content of skeletal muscle with immobilization, which may negatively impact its force generation.

magnetic resonance spectroscopy; muscle strength; skinned fibers

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In addition, muscle biopsies obtained in a subset of patients were prepared for single-fiber in vitro force mechanics.

METHODS

Subjects

Sixteen individuals (7 men, 9 women; mean age 38 ± 3 yr) who sustained a unilateral ankle malleolar fracture and were subsequently treated by open reduction-internal fixation and thirteen healthy subjects (7 men, 6 women; mean age 33 ± 3 yr) participated in this study. After surgery, patients were immobilized in a short leg cast for 6–8 wk, hereafter referred to as 7 wk of immobilization. After cast immobilization, a subset of immobilized subjects (n = 8) participated in a 10-wk rehabilitation program (three 1-h training sessions/wk) that focused on both strength and endurance as described previously (51). All participants were informed of the purpose of the investigation and gave their informed consent. All experimental procedures were approved by the Institutional Review Boards at the University of Florida and the University of Pennsylvania.

Experimental Protocol

31P-MRS, MRI, and isometric muscle strength testing were performed in healthy and immobilized subjects. In the immobilized subjects, measurements were performed at 0 wk (n = 16), 5 wk (n = 8), and 10 wk (n = 8) of rehabilitation. To protect against iatrogenic injury, 0-wk rehabilitation measurements were acquired 1 wk postimmobilization but before the start of rehabilitation. Additionally, in a subset of patients (n = 6), muscle biopsy samples were acquired from the medial gastrocnemius muscle at the time of surgery to perform the single-fiber force mechanics.

Magnetic Resonance Spectroscopy

31P-MRS experiments were performed at rest on the medial gastrocnemius muscle using a 1-m bore, 2.0-T superconducting magnet, interfaced with a home-built spectrometer (51) and a single-turn 4 cm × 6 cm oblong surface coil, double tuned to both 31P and 1H frequencies. The subjects were placed in a supine position such that the upper one-third of the medial gastrocnemius muscle was centered over the coil. Spectra were collected by using an adiabatic 90° pulse with a sweep width of 3 kHz and 1,024 complex data points. The homogeneity of the magnetic field was adjusted using the proton signal from water. Water line widths were typically 0.18–0.30 ppm. The pulse repetition time was set at 30 s, and the signal was averaged for 18 min. The spectra were manually phased, and the areas of the γ-ATP, Pi, and PCR peaks were integrated by use of customized software (50). Intracellular pH was calculated from the chemical shift of the P, peak, based on the equation pH = 6.75 + log [(δ - 3.27)/(5.69 - δ)], where δ is the chemical shift of the P, peak (in ppm) relative to PCR. Resting phosphate concentrations were calculated by assuming a resting ATP concentration of 8.2 mmol/l (21). ATP levels have been reported to be unchanged in human muscles after immobilization (20, 23, 31).

Magnetic Resonance Imaging

Proton magnetic resonance images were obtained with a three-dimensional fast gradient echo imaging sequence using a 1.5-T magnet (Signa; General Electric Medical Systems, Waukesha, WI) and standard extremity quadrature coil. The data were acquired in three series to cover the total region from the midthigh to the calcaneus. Each series employed an encoding matrix of 256 × 256 × 28, a field of view of 16 × 16 × 19.6 cm, a pulse repetition time of 51 ms, and an echo time of 10 ms. Chemically selective fat suppression was used to enhance the definition between muscle groups. The fat-free maximal muscle CSA of the primary ankle plantar flexors (soleus, medial gastrocnemius, and lateral gastrocnemius) was determined by using a custom-designed interactive computer program as described earlier (15). The sum of the maximal CSA of the individual muscles was defined as the plantar flexor CSA.

Strength Assessment

Ankle strength testing was performed bilaterally in the immobilized subjects and unilaterally in the healthy control subjects (right leg). Plantar flexor peak torque was measured with a Biodex isokinetic dynamometer (Biodex Medical Systems, Shirley, NY) as described earlier (40). Peak torque was assessed at 0° plantar flexion as measured with a standard goniometer from a neutral position (90° angle between the fibula and calcaneus). The subject’s hips were flexed to 90–100° and the knee was flexed to 10°. Subjects performed three isometric contractions (5-s contractions separated by 30 s of rest). In cases with >10% of intertrial variation, the subject was given a 2-min rest period before the testing procedure was repeated. The highest plantar flexor peak torque was determined after correction for baseline torque. Specific plantar flexor torque was calculated by dividing the plantar flexor peak torque by the plantar flexor CSA (determined by MRI). Note that although 31P-MRS measurements were localized to the medial gastrocnemius muscle, strength was measured from all the plantar flexor muscles.

Muscle Biopsies

Muscle biopsies were acquired from the medial gastrocnemius muscle using the needle biopsy technique as described previously (16, 49). The skin of the calf muscles was cleaned and disinfected. After the area was sterilized, a local anesthetic was injected into a small area of the calf. A small incision (less than 1/4 in.) was made to insert the needle and to take approximately a 60–100 mg muscle tissue under suction. After the biopsy was taken, the incision was closed using a Steri-Strip bandage and a sterile dressing was placed over the site.

Single Fiber Measurements

Permeabilized fiber preparation. Small bundles of muscles were dissected and tied to a Teflon stick (to maintain resting in vivo fiber lengths). The bundles were first immersed in solution A (170 mM potassium propionate, 5 mM EGTA, 2.5 mM MgCl2, 2.5 mM ATP, 10 mM imidazole, 0.2 mM PMSF, 50 μM leupeptin, 50 μM antipain, pH 7.0) at 4°C overnight and subsequently stored in solution B (170 mM potassium propionate, 5 mM EGTA, 2.5 mM MgCl2, 2.5 mM ATP, 10 mM imidazole, 1 mM NaN3, 2.5 mM glutathione, 50% glycerol, pH 7.0) at 4°C for 4 h and then at −20°C for longer storage. This skinnng procedure is essentially the same as that described by Eastwood et al. (14) and results in loss of endogenous calmodulin, myosin light chain kinase, and myosin light chain phosphatase activities (43).

Solutions. The force-Pi relationship was determined in activating and relaxing solutions at varying [Pi] levels. The relaxing solutions contained 100 mM free K+, 51 mM free Na+, 1 mM free Mg2+, 100 mM TES, 25 mM EGTA, 5 mM ATP, 20 mM creatine phosphate, and 10 mM glutathione, pH 7.1. The total ionic strength of this solution was 0.2 M. The activating solution was essentially the same, with the addition of 0.048 mM free Ca2+, which is equivalent to about pCa 4.3 (29). The various Pi solutions were prepared by proportionally mixing activation solutions with and without 20 mM Pi, and relaxing solutions with and without 20 mM Pi. The solution constituents and mixing were calculated for 20°C, based on a solution calculation computer program kindly provided by Dr. Yale Goldman. The solution temperature was controlled by an Isotemp refrigerated circulator (model 9100, Fisher Scientific).

Experimental apparatus and contractile protocol. The mechanical setup used for this experiment is essentially the same setup as that used in other published studies (29, 44). A single-fiber segment was isolated in solution B under a dissecting microscope and mounted...
between the force transducer and motor lever arm in relaxing solution (pCa 8). The entire setup was mounted on the stage of a microscope, equipped with a ×40 (0.75 NA) objective and Nomarski interference contrast optics, for direct observation of sarcomere patterns and determination of sarcomere length and fiber width. The force-Pᵢ relationships were determined at 20°C by first incubating the fiber segments in the relaxing solution of a specific [Pᵢ] to allow Pᵢ to be equilibrated inside the fiber and then activating the fiber segments in the activating solution at a resting sarcomere length. The fibers were randomly cycled through activating and relaxing cycles at different Pᵢ levels (0, 4, 10, 16, and 20 mM Pᵢ). After isometric tension measurements were performed the fiber CSA was determined. Because the permeabilized fiber segments were mounted on the stage of a light microscope, it was easy to measure the widths of the fibers. The depths of the fibers were determined optically by focusing the top of the fiber segment and then the bottom of the fiber segment. The CSA was assumed to be elliptical and calculated accordingly (19). Finally, the fibers were saved for fiber typing by silver-stained one-dimensional-gel electrophoresis to determine myosin heavy chain isoform composition (47). Data from type I fibers were pooled (5–10 fibers for each Pᵢ level in each subject) and are presented in this work.

**Force-pCa measurements.** To ensure that the Ca²⁺ levels (pCa 4.3) used in the in vitro experiments above were sufficient to ensure near full-activated Ca²⁺ tension, the force-pCa relationship was determined in control and immobilized muscle fibers (one subject). The force-pCa relationship was determined in solutions ranging in Ca²⁺ concentration from 0.01 μM (pCa 8.0, relaxing solution) to 10 mM (pCa 4.3, full activating solution) (29) at three different [Pᵢ] levels: 0, 5, and 8.5 mM. The pCa-force data were fit to the Hill’s equation: $P = \frac{P_0}{1 + 10^{\alpha (pK - pCa)}}$, where $P_0$ represents the isometric tension at full Ca²⁺ activation (amplitude), $pK$ is the pCa that gives 0.5 $P_0$ (midpoint), and $n$ is the Hill coefficient (slope) (35).

**Reliability Assessment**

To determine the reproducibility of the ³¹P-MRS measurements, repeated measurements were performed in six healthy, control subjects over the same time frames (0, 5, and 10 wk) as performed in the immobilized subjects. The intraclass correlation coefficients (2,1) were calculated for the primary ³¹P-MRS measures ($P_i = 0.81–0.89$; $P_i/PCr = 0.80–0.81$).

**Data Analysis**

All statistical analyses were performed with SPSS for Windows, version 11.0.1 (SPSS, Chicago, IL). All the results are expressed as means ± SE. Independent $t$-tests were used to compare the ³¹P-MRS and isometric torque measurements between control ($n = 13$) and immobilized subjects at 0 wk ($n = 16$) and 10 wk of rehabilitation ($n = 8$). In the group of immobilized subjects who received rehabilitation ($n = 8$), changes in phosphate metabolites and torque were assessed with a repeated-measures ANOVA model, followed by a Bonferroni-Dunn post hoc test. Research hypotheses were tested at an α level of 0.05.

Regression analysis was performed to assess the correlation between $P_i$ content and specific plantar flexor torque in the immobilized subjects. Also multiple linear regression was used to determine how resting $P_i$ content and CSA together account for changes in plantar flexor torque in immobilized subjects. For this purpose, isometric plantar flexor torque from the involved leg was normalized to that of the uninvolved leg. Data of all the immobilized subjects at each time point (0, 5, and 10 wk of rehabilitation) were pooled together. Significance level was set at 0.05.

**RESULTS**

**Magnetic Resonance Spectroscopy**

After immobilization, the resting $P_i$ content (8.41 ± 0.41 vs. 5.07 ± 0.33 mM; $P < 0.001$) and the $P_i/PCr$ ratio (0.22 ± 0.03 vs. 0.12 ± 0.01 mM; $P < 0.001$) were significantly elevated in immobilized subjects when compared with control subjects (see Figs. 2 and 3). At 0 wk rehabilitation, the $P_i$ content and the $P_i/PCr$ ratio were elevated by 66 and 83%, respectively, compared with control values. However, the PCr concentration (38.56 ± 1.88 vs. 40.53 ± 1.63 mM; $P = 0.43$) and the basal pH (7.06 ± 0.04 vs. 7.03 ± 0.01; $P = 0.21$) values were not significantly different between the two groups.

In immobilized subjects from whom data were acquired during rehabilitation, the $P_i$ content and the $P_i/PCr$ ratio progressively decreased during the 10 wk of rehabilitation, resulting in values similar to those of control subjects at the end of rehabilitation ($P_i$: 5.92 ± 0.33 mM; $P = 0.90$; $P_i/PCr$ ratio: 0.14 ± 0.01, $P = 0.87$; Figs. 1–3). The greatest decrease in the $P_i$ content (80%) and the $P_i/PCr$ ratio (75%) was observed during the first 5 wk of rehabilitation. Significant differences were noted between the values at 0 wk and 5 wk of rehabilitation for both the $[P_i]$ ($P < 0.001$) and the $P_i/PCr$ ratio ($P < 0.001$).
significant differences between immobilized subjects at 0 and 5 wk rehab \((P < 0.05)\). \(*\)Statistically significant differences between healthy control subjects and immobilized subjects at 0 wk rehab \((P < 0.05)\). \(^\dagger\)Statistically significant differences between immobilized subjects at 0 and 5 wk rehab \((P < 0.05)\). \(^\ddagger\)Statistically significant differences between immobilized subjects at 0 and 10 wk rehab \((P < 0.05)\).

Fig. 3. \(P_i\)-to-phosphocreatine ratio \((P_i/PCr)\) measured in the medial gastrocnemius muscle of healthy control subjects \((n = 13)\) and immobilized subjects at 0 wk \((n = 16)\), 5 wk \((n = 8)\), and 10 wk \((n = 8)\) of rehabilitation. Values are means \pm SE. \(*\)Statistically significant differences between healthy control subjects and immobilized subjects at 0 wk rehab \((P < 0.05)\). \(^\dagger\)Statistically significant differences between immobilized subjects at 0 and 5 wk rehab \((P < 0.05)\). \(^\ddagger\)Statistically significant differences between immobilized subjects at 0 and 10 wk rehab \((P < 0.05)\).

However, from 5 wk to 10 wk of rehabilitation, no significant differences were observed in the \(P_i\) content \((P = 0.43)\) and the \(P_i/PCr\) ratio \((P = 0.07)\), although the average values dropped by an additional 20 and 25\% for the \(P_i\) content and the \(P_i/PCr\) ratio, respectively. No significant changes were observed in the PCr content and the basal pH throughout the 10 wk of rehabilitation.

**Strength Assessment**

Not surprisingly, comparisons between control \((3.16 \pm 0.18 \text{ Nm/cm}^2)\) and immobilized subjects at 0 wk of rehabilitation \((1.82 \pm 0.18 \text{ Nm/cm}^2)\) revealed significant differences in specific plantar flexor torque \((P = 0.001)\). The specific torque in immobilized subjects was 42\% lower than that of healthy control subjects.

During the course of rehabilitation, immobilized subjects demonstrated significant gains in specific torque. Similar to the \(^{31}\)P-MRS findings, the greatest recovery in specific torque occurred during the first 5 wk of rehabilitation, with an 80\% increase \((P = 0.001)\). From 5 wk to 10 wk of rehabilitation, no significant differences were observed in the specific torque \((P = 0.45)\), although the average values were \(\sim 20\%\) higher. Also, although the immobilized subjects showed improvements in specific force production, at 10 wk of rehabilitation significant differences still existed between the specific torque of immobilized \((2.79 \pm 0.19 \text{ Nm/cm}^2, P = 0.12)\) and control subjects. Figure 4 shows the changes in specific plantar flexor torque during the 10 wk of rehabilitation.

Regression analysis showed an inverse relationship between specific plantar flexor peak torque and the resting \(P_i\) content in the immobilized subjects \((r = 0.65, P < 0.01)\). Figure 5 provides the results of the regression analysis. Furthermore, during the course of rehabilitation plantar flexor CSA only accounted for 39\% of the variance in torque \((P < 0.01)\), whereas CSA and the resting \(P_i\) content together accounted for 52\% of the variance in torque in these subjects \((P < 0.01)\).

**Single Fiber Measurements**

As expected, elevation of \([P_i]\) significantly depressed the force production in human skeletal muscle fibers. Figure 6 shows the relationship between peak tension and \([P_i]\) in type I human muscle fibers obtained from the medial gastrocnemius \((n = 6)\). All data were normalized to the maximal peak tension observed in solution in the absence of \([P_i]\) \((0 \text{ mM} \ P_i)\). Force measurements at each \([P_i]\) were measured in at least 5–10 fibers in all six subjects; the average force and specific force data are provided in Table 1.
The findings of Millar and Homsher (35), Pi affects all three performed in one subject, also show that, in agreement with the postimmobilization time points. These data, although only saturated Ca$^{2+}$ levels in human muscle fibers can be considered maximal and at 4.3 in vitro force production in both control and immobilized human muscles ranging between 7 and 4.3 clearly demonstrate that at a pCa force production by

Finally, the force-pCa measurements performed at pCa levels ranging between 7 and 4.3 clearly demonstrate that at a pCa of 4.3 in vitro force production in both control and immobilized human muscle fibers can be considered maximal and at saturated Ca$^{2+}$ levels. Figure 7 shows the isometric force-pCa curves at three different [Pi] levels at preimmobilization and postimmobilization time points. These data, although only performed in one subject, also show that, in agreement with the findings of Millar and Homsher (35), Pi affects all three parameters that define the force-pCa relationship ($n$, $pK$, and $P_0$). Of interest to note is that after immobilization maximal tension was reduced and a clear rightward shift of the force-pCa curve was observed.

**DISCUSSION**

A clear understanding of the pathophysiological processes underlying the loss of muscle strength with disuse is lacking. Although factors such as contractile area and neural drive are purported to explain the loss of strength, they cannot completely account for the weakness that results from disuse (4). There is substantial evidence from skinned fiber studies in mammalian muscle that elevated levels of Pi inhibit muscle force (6, 9, 11). Specifically, these studies have shown that Pi inhibits actomyosin cross-bridge cycling and suppresses muscle force via a shift in the force-Ca$^{2+}$ curve (25). The purpose of this study was to quantify changes in the resting energy-rich phosphate content, specifically Pi, and specific skeletal muscle force production after cast immobilization. On the basis of our findings, it is evident that skeletal muscle disuse during cast immobilization causes a change in the specific plantar flexor torque and the Pi content of human skeletal muscle. Specifically, we found a 42% decrease in specific torque and a 66% increase in the [Pi] of immobilized muscles compared with control muscles. In a group of eight immobilized subjects monitored during rehabilitation, the Pi content returned to control values during the course of 10 wk of rehabilitation, with 80% of the recovery occurring in the first 5 wk of rehabilitation. No significant changes were noted in the intracellular pH or Pi concentration throughout the entire protocol. Force measurements in permeabilized single human muscle fibers further revealed a nonlinear relationship between relative force and Pi with a F$^{50}$ value of ~16 mM at 20°C. Most importantly, in vitro force mechanics data suggest that varying the Pi levels in human skeletal muscle within the ranges observed across individuals in vivo (4–10 mM) may vary force production by ~16%.

A large number of studies have shown that transient changes in Pi levels provide an important pH buffer and regulate mitochondrial function. Chronically elevated resting levels of Pi have been observed during inflammation (2, 30), during denervation (27, 56), and in a number of myopathies (3, 7, 45). Additionally, a high Pi/PCr ratio (an indirect measure of the phosphorylation potential) is considered to be a marker of nonspecific muscular damage and is well documented in exercise-induced injury as well as in neuromuscular diseases (34). In most cases elevated Pi levels are associated with a corresponding decrease in the PCr content (23). Despite equivalent, but opposite, absolute changes in the PCr(∼2 mM) and Pi (+3.3 mM) concentration, this study lacked the sensitivity to detect a significant change in the resting PCr concentration. This is most likely a reflection of the 10-fold higher resting PCr concentration compared with the [Pi], which results in smaller relative changes (5 vs. 66%).

As mentioned earlier, an elevated Pi content and Pi/PCr ratio has previously been noted in both humans and animals with denervation atrophy (27, 56). The denervation atrophy model is comparable to the cast immobilization model in that both models are associated with decreased neuromuscular activity and result in muscle atrophy. After nerve crush denervation in the gastrocnemius and soleus of rats, Lai et al. (27) observed a 54% increase in the Pi/PCr ratio, which decreased progres-

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**Fig. 6.** Relationship between [Pi] in solution and relative force production in single skinned human muscle fibers in immobilized subjects ($n = 6$) at the time of surgery. Values are means ± SE.

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**Table 1.** Force and force/cross-sectional area values in type I human muscle fibers at varying Pi concentrations as measured by using single-fiber force mechanics

<table>
<thead>
<tr>
<th>Pi Concentration (mM)</th>
<th>0</th>
<th>4</th>
<th>10</th>
<th>16</th>
<th>20</th>
</tr>
</thead>
<tbody>
<tr>
<td>Force, kN $\times 10^{-9}$</td>
<td>592 ± 104</td>
<td>399 ± 70</td>
<td>332 ± 66</td>
<td>287 ± 68</td>
<td>267 ± 71</td>
</tr>
<tr>
<td>Force/cross-sectional area, kN/m²</td>
<td>73.9 ± 6.66</td>
<td>53.69 ± 3.87</td>
<td>44.36 ± 3.10</td>
<td>37.65 ± 3.54</td>
<td>34.34 ± 3.96</td>
</tr>
</tbody>
</table>

Values are means ± SE ($n = 6$).
that some of the elevation in the Pi content and Pi/PCr ratio increased 1–2 days after reloading. Therefore, it is plausible not change immediately after 5 wk of complete bed rest but muscle damage, in the lower limbs of normal male subjects did netic resonance proton relaxation time, a marker to assess similarly, LeBlanc et al. (28) noted that the transverse (T2) mag-

Fig. 7. Force-pCa relationship at varying Pi levels (0, 5, and 8 mM) in single skinned human muscle fibers from 1 subject before immobilization (Pre IM) and after 7 wk of immobilization (Post IM). Values are means ± SE. All tensions are scaled to the fiber tension at pCa of 4.3 at 0 mM Pi at preimmo-
bilization. Each point is the average of 4–6 fibers. The sigmoidal curves represent the least-squares fit of the data to the Hill’s equation.

sively to reach normalized values at 7 wk postinjury. In contrast, Zochodne et al. (56) found no change in the phosphate content of the forearm muscles of 4 immobilized subjects after 6 wk of cast immobilization. The lack of metabolic change in this study could be due to the fact that forearm muscles typically do not demonstrate a large degree of muscular atrophy or because 31P-MRS measurements were performed within 2 h after removal of the cast. In contrast, 31P-MRS measure-
ments in the present study were performed after 1 wk of reloading after immobilization. Therefore, we cannot distin-
guish whether the increase in the phosphate metabolites ob-
served in our study is due to immobilization or reloading.

There is substantial evidence from animal suspension studies that reloading induces significant muscle damage in the un-
loaded hindlimb muscles (26, 42). Krippendorf and Riley (26) showed that compared with measurements performed a few hours after suspension, muscles exposed to 12–48 h of reloading demonstrate significant myofiber swelling, interstitial edema, macrophage activation, and monocyte infiltration. Similarly, LeBlanc et al. (28) noted that the transverse (T2) mag-
netic resonance proton relaxation time, a marker to assess muscle damage, in the lower limbs of normal male subjects did not change immediately after 5 wk of complete bed rest but increased 1–2 days after reloading. Therefore, it is plausible that some of the elevation in the Pi content and Pi/PCr ratio observed in this study is due to reloading-induced muscle injury and not disuse per se.

The elevated Pi content with disuse observed in this study is of significant clinical relevance, as it may have important functional consequences. In our patient population, we found a significant relationship between the resting [Pi] and specific plantar flexor torque ($r = 0.65, P < 0.01$). These results were further supported by our findings that during the course of rehabilitation plantar flexor CSA only accounted for 39% of the variance in torque ($P < 0.01$), whereas changes in CSA and resting Pi content together accounted for 52% of the variance in torque ($P < 0.01$). Collectively these findings support the contention that after immobilization changes in energy-rich phosphates may contribute to the change in force-generating capacity of skeletal muscle.

We confirmed our findings by performing single-fiber mea-
surements in a subset of patients. To our knowledge, this is the first time that the force-depressing effect of [Pi] has been studied in human single-fiber muscles. Our single-fiber studies showed a nonlinear relationship between [Pi] and peak tension, with an apparent F50 of ~16 mM. Interestingly, our data suggest that varying the Pi levels in human skeletal muscle within the ranges observed across individuals in vivo (4–10 mM) may vary force production by ~16%. Note that the average resting [Pi] in the immobilized patients at 0 wk of rehabilitation was 8.41 ± 0.41 mM, compared with 5.07 ± 0.33 mM in healthy controls. Previous in vitro force mechanics measurements performed in other mammalian (rabbit psoas) muscle fibers have reported a $k_i$ (phosphate concentration at which force depression is half maximum) ranging from 3 to 12 mM (9, 46, 54). The fact that our F50 values were slightly higher may be due to either species or fiber type differences (17, 36–38, 41, 46). It should also be noted that because of the reported high risk of fiber deterioration at high temperatures (10, 12), single-fiber studies investigating the impact of specific metabolites on force production, including the present study, have been performed at temperatures well below 37°C. However, some studies show that the impact of Pi on force development is temperature dependent (10–12), such that higher and more physiological temperatures show a smaller force dependence on Pi.

The precise mechanism by which Pi inhibits force generation is unclear. Numerous studies of muscle mechanics in skinned, mammalian, skeletal muscle fibers have shown that increasing the [Pi] decreases isometric tension (6, 9), while having little effect on unloaded shortening velocity (9). An elevated resting [Pi] has also been shown to increase fatigue resistance during repeated maximal contractions (17, 52). The decrease in iso-
metric tension with an increase in Pi is largely attributed to its effect on the cross-bridge cycling. Increased levels of Pi presumably reduce the number of cross bridges in the force-
developing state and therefore result in a decrease in isometric tension (8). Furthermore, Brandt et al. (5) have shown that, in skinned fibers, Pi has an effect not only on force generation but also on the Ca2+ sensitivity of the myofilibrils. In agreement with these observations, Kentish (25) reported that an increase in [Pi] over the range 0–20 mM decreases maximum Ca2+-
regulated force and shifts the force-Ca2+ relationship to higher concentrations. Also, our findings clearly indicate that increasing [Pi] results in a rightward shift of the force-pCa relationship with an increase in the mean Ca2+ required for 50% activation of the muscles. To eliminate the effect of Pi on Ca2+ sensitiv-
ity, we performed in vitro experiments at a pCa of 4.3, i.e., at near fully activated Ca2+ activated tension (36). We also confirmed that the Ca2+ level used in the in vitro experiments (pCa 4.3) was sufficient to ensure near fully activated Ca2+ tension in control as well as immobilized human muscle at varying [Pi] levels.

In the present study, 31P spectra were localized by use of a surface coil placed over the skin covering the region of interest, i.e., the medial gastrocnemius muscle. We previously deter-
mied the sensitive volume of the surface coil employed in the present study and showed that >95% of the signal detected by the coil is sampled from the medial gastrocnemius muscle (39). Therefore it is very unlikely that the increase in $P_i$ content after cast immobilization is due to the fact that we may be sampling more from the soleus muscle, which has a higher proportion of slow-twitch fibers. Furthermore, using an image-guided multi-volume localization technique, we previously measured the $P_i$/PCR ratio in healthy subjects and found a $P_i$/PCR ratio of 0.12 ± 0.01 in the medial gastrocnemius and 0.15 ± 0.01 in soleus muscles (49). In the present study, the $P_i$/PCR ratio measured from the medial gastrocnemius in healthy subjects is 0.12 ± 0.01 and is in agreement with the values reported in the literature (49). Additionally, after immobilization, the immo-
obilized subjects showed an increase in the $P_i$/PCR ratio to 0.22 ± 0.03, which is much greater than the $P_i$/PCR ratio measured from the soleus muscle in healthy adults. Therefore, the reported shift in the $P_i$/PCR ratio with limb disuse is not due to sampling of more slow-twitch fibers, because the contribution of the signal from the soleus muscle is small; and, moreover, even if the soleus muscle contributed to the signal, the difference in $P_i$ content of fast- and slow-twitch healthy human muscles is small (49).

In conclusion, our data clearly demonstrate a change in the resting energy-rich phosphate content of skeletal muscle with disuse, which may negatively impact its force-generating capacity. The mechanisms underlying the increase in $P_i$ content after cast immobilization are not clear and may include reloading-induced muscle damage. As future endeavors are directed toward understanding the mechanism of loss of strength after disuse, the role played by metabolic factors merits further inquiry.

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