

REINNERVATION AND REGENERATION OF DENERVATED RAT SOLEUS MUSCLES

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Muscle atrophy is a serious and sometimes irreversible consequence of peripheral nerve damage.⁴ Recently, we found that muscle atrophy is reversible to varying extents when denervated muscles are grafted with nerve implants.⁴ In that study, rat extensor digitorum longus (EDL) muscles were denervated for 1–12 months. The muscles were then grafted into innervated sites of host animals. The grafting operation resulted in a significant increase in mass and force-generating ability of the muscles over the denervated state; the degree of improvement was inversely proportional to the time of initial denervation. One question to arise from the study was whether or not grafting contributed to the functional return of the denervated muscles or if nerve implantation alone accounted for the return. To answer this, soleus muscles were denervated and then reinnervated with and without concomitant grafting. The soleus, unlike the EDL, can be locally denervated and reinnervated without disturbing its tendons. This study found that 2-month denervated muscles grafted with nerve implants have a higher mass and generate twice the force of that generated by denervated muscles which receive nerve implants but are not grafted.

METHODS

Experiments were performed on 34 highly inbred, conventionally held, 3 1/2–4-month-old, male WI/HicksCar rats. The rats were anesthetized with ether during surgery and sacrificed with ether overdose.

Experiments were approved by the University Committee on Use and Care of Animals.

Four groups of denervated muscles were used in this study. In all groups, the soleus muscles were denervated for 2 months. In the first group, the muscles were denervated by cutting the sciatic nerve high in the thigh region and then directing the cut ends of the nerve into nearby muscles. This method is highly effective in maintaining the denervated state of the crural muscles.⁴ In the remaining three groups, the soleus muscles of each animal were locally denervated by the following procedure. The nerve branches innervating each soleus muscle were ligated, cut, and marked with powdered carbon. With a needle attached to the ligating suture, the ligated end of the nerve branches was pulled through adjacent muscles and then sutured to the lateral aspect of the biceps femoris. After 2 months, the first group of the locally denervated soleus muscles was removed for comparison with the soleus muscles that were denervated by sciatic nerve section.

In the remaining two denervated groups, each soleus muscle was reinnervated by implanting its original nerve into the muscle. Each nerve was cut free of its attachment to the biceps femoris and then implanted into the soleus muscle with forceps. In one of these two groups, the soleus muscles were orthotopically autografted prior to receiving nerve implants. In the other group, the soleus muscles received nerve implants but were not grafted. Controls of these groups were age-matched, normal intact soleus muscles.

Physiology. Contractile property measurements were performed in vitro according to the methods

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of Brooks and Faulkner.² The tendons of each soleus muscle were ligated with suture and then cut. The muscles were immersed in a CO₂-buffered, oxygenated mammalian Krebs's solution maintained at 25 ± 0.3°C. One tendon of each muscle was attached to a fixed post and the other to a force transducer on-line with a Macintosh computer. Customized software (LabView, National Instruments, Austin TX) reported force and time in milliNewtons (mN) and milliseconds (ms), respectively. The muscles were stimulated by two platinum field electrodes with supramaximal voltage and square-wave pulses of 0.2-ms duration for twitch tension and 0.7-ms duration for tetanic tension. For tetany, the muscles were stimulated at 20–140 Hz in 20-Hz increments. The variables measured were maximum twitch tension (P_t), maximum tetanic tension (P_o), and the optimal length (L_o) for tension development.⁶ Muscle lengths were measured at L_o. Fiber lengths (L_f) were estimated based on an L_f/L_o ratio of 0.62.¹⁵ The cross-sectional area (CSA) was estimated from mass, fiber length, and a muscle density of 1.06.⁶ Specific force (specific P_o), reported as kiloNewtons/meter² (kN/m²), was determined as P_o/CSA. After measurements were made, the muscles were removed from the bath, weighed, and frozen in a dry ice/isopentane mixture.

Statistical Analysis. Statistical analyses were performed with SAS/STAT software (Cary, NC) and included one-way analysis of variance, post hoc analysis by the Duncan–Waller multiple range test, Student's *t*-test, and Tukey's student range test and nonparametric analysis by the Wilcoxon and Kolmogorov–Smirnov tests. Significance levels were set a priori at *P* < 0.05.

Histology. Soleus muscles, which had been locally denervated for 2 months, were cryosectioned longitudinally at 25 μm and stained with acetylcholinesterase–silver stain to control for the absence of nerve fibers.⁹ Approximately 2-mm-thick sections through the belly of each remaining muscle were cut perpendicular to the angle of muscle fiber pennation, fixed in 4% paraformaldehyde, and embedded in glycol methacrylate. These muscles were cross-sectioned at 2 μm, stained with Gill's hematoxylin and phloxine–eosin,¹⁴ and examined with light microscopy.

RESULTS

Physiology. The values for the mean mass, P_t, P_o, and P_t/P_o of the locally denervated soleus muscles were slightly, yet significantly, higher than muscles denervated by sciatic nerve section, yet there was no

significant difference in the specific P_o between the two groups of muscles (Table 1). The mean mass of soleus muscles denervated for 2 months was approximately 35% of that for the control, normal intact soleus muscles. The mean P_o generated by the locally denervated muscles was less than 2% of that generated by the controls.

There was no significant difference in the mean values of the mass between nerve-implanted denervated muscles and the grafts of nerve-implanted denervated muscles (Table 1). There were, however, significant differences in the mean forces between these groups of muscles. The mean values for P_t, P_o, and specific P_o of the grafted muscles were twice those of the nongrafted muscles. These values for the grafts of denervated muscles were not significantly different from those of the grafts of normal muscles.

Histology. Local denervation was effective in maintaining the denervated state of the soleus muscles for the 2-month study period. Silver-staining of the locally denervated muscles revealed only an occasional nerve fiber, and end-plates, including ectopic ones, were not present in any of the denervated muscles.

Experimental muscles were comprised of two fiber types, those which were similar in size to those in control muscles and small fibers which resembled the atrophic fibers of the 2-month denervated muscles. The grafted muscles contained many muscle fibers with centrally located nuclei, indicating that the fibers had undergone regeneration.³ The muscles which received nerve implants but were not grafted were comprised primarily of fibers with peripherally located nuclei.

DISCUSSION

Denervated muscles which are grafted with nerve implants generate significantly more force than denervated muscles which receive nerve implants but are not grafted. The major difference between the muscles is that fibers within the grafted muscles degenerate and regenerate, whereas those in the nongrafted muscles do not. The reason why muscle fiber regeneration accompanying reinnervation confers an advantage over reinnervation alone is not understood. However, regenerating muscle fibers reexpress a number of molecules present at myogenesis and synaptogenesis. These include the myogenic regulators MyoD and myogenin,^{8,10} the cell adhesion molecules N-CAM,^{5,12} tenascin,^{11,13} M-cadherin,^{1,13,16} and N-cadherin,⁵ and the motor end-plate-associated molecule, agrin.¹⁷ The presence of these molecules in regenerating muscles may play a role in the greater

Table 1. Masses and contractile properties of control and experimental muscles.

| Muscle type | mass (mg) | P _i (mN) | P _o (mN) | P _i /P _o | Specific P _o (kN/m ²) |
|---|-------------|---------------------|---------------------|--------------------------------|--|
| Control (<i>n</i> = 24) | 164.5 ± 3.5 | 348.8 ± 9.8 | 1505.3 ± 34.3 | 0.23 ± 0.00 | 159.5 ± 4.1 |
| Sciatic section (<i>n</i> = 10) | 45.3 ± 3.1 | 5.1 ± 1.4 | 8.9 ± 2.1 | 0.56 ± 0.04 | 2.9 ± 0.7 |
| Local denervation (<i>n</i> = 6) | 56.7 ± 3.4* | 15.7 ± 3.1* | 20.3 ± 4.3* | 0.83 ± 0.08* | 4.0 ± 0.9 |
| 2-month denervated, nerve implant (<i>n</i> = 8) | 79.6 ± 6.1 | 53.8 ± 12.0 | 203.4 ± 33.6 | 0.25 ± 0.04 | 34.5 ± 6.5 |
| 2-month denervated, nerve implant + grafted (<i>n</i> = 8) | 79.4 ± 5.0 | 114.6 ± 14.8† | 437.1 ± 66.1† | 0.28 ± 0.05 | 75.4 ± 10.3† |

All values given as means ± SEM (standard error of the mean).

*Represents significant differences between 2-month denervated soleus muscles denervated by sciatic nerve sectioning and locally denervated soleus muscles (*P* ≤ 0.05).

†Represents significant differences between 2-month denervated nongrafted and grafted muscles (*P* ≤ 0.05).

reversal of atrophy in grafted versus nongrafted denervated muscles.

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