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Muscle Fiber Type Composition and Morphometric Properties of Denervated Rat Extensor Digitorum Longus Muscle

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Aim. Analysis of fiber type composition and fiber size of extensor digitorum longus (EDL) muscle in rats during 30 days of denervation.

Methods. Fiber types were defined immunohistochemically using monoclonal antibodies specific for slow (type I) and fast fibers (type IIA, IIX, and IIB). Antibodies were applied on transverse sections of denervated extensor digitorum longus muscles, 12, and 30 days after denervation. Cross sectional area of muscle fibers was analyzed morphometrically by computerized image analysis.

Results. Control EDL muscle was composed of 41% of type IIB, 32% of IIA, 23% of IIX, and 4% of type I muscle fibers. The most profound effect of denervation was observed 30 days after the transection of the sciatic nerve. Denervation decreased the percentage of both type IIB and type IIX muscle fibers to 36% and 13% of the control muscle, respectively ($p<0.001$ for both), and increased the percentage of types IIA and type I muscle fibers to 42% and 9% of the control, respectively ($p<0.001$ for both). Morphometric analysis revealed progressive atrophy of all fast muscle fibers, which started 6 days after denervation ($p<0.001$). Thirty days after the sciatic nerve transection, a strong reduction in fiber size of type IIA, IIX, and IIB muscle fibers was observed ($p<0.001$ for each type). Type I muscle fibers initially showed the reduction in fiber size ($p<0.001$) but regained the size of control fibers until day 30.

Conclusion. Denervation decreased the percentage of type IIX and IIB muscle fibers, with concomitant increase in type IIA and type I muscle fibers. The reduction in fiber size was observed in type IIA, IIX, and IIB muscle fibers.

Key words: *immunohistochemistry; muscle denervation; muscle fibers; muscular atrophy; skeletal muscle*

In adult mammals, four different types of skeletal muscle fibers can be identified by use of specific monoclonal antibodies to myosin heavy chain isoforms (1): slow-contracting fibers, called type I, containing the slow myosin heavy chain (β/slow-myosin heavy chain), and 3 different types of fast-contracting fibers, called IIA, IIX, and IIB, which express IIA-, IIX-, and IIB-myosin heavy chains, respectively.

Skeletal muscle fibers are dynamic structures capable of altering their pattern of myosin gene expression. Fiber type composition of adult muscles can rapidly change in response to denervation (2), electrical stimulation (3), hormones (4), overloading (5), and unloading (6). Among these factors, innervation seems to be crucial for maintenance and plasticity of fiber type-specific profiles (7-9).

The role of innervation has been investigated also by denervation experiments (10-15). By using muscle regeneration model in adult rats, it has been shown that acquisition of a slow myosin heavy chain mRNA profile depends on the presence of a slow nerve, whereas the appearance of an adult fast myosin heavy chain mRNA does not depend on innervation (2).

The absence of nerve impulses in fast extensor digitorum longus muscle during denervation leads to the progressive atrophy of skeletal muscle fibers (11,12). Most previous studies used the myofibrillar adenosine triphosphatase (mATPase) histochemistry to classify muscle fiber types I, IIA, and IIB (16). However, type IIX muscle fibers could not be successfully delineated by that method, so the knowledge about the changes in fiber type IIX has not been sufficient. The aim of this study was to explore the changes in fiber distribution and fiber size of all muscle fiber types. In order to investigate the expression pattern of all myosin heavy chain isoforms in denervated extensor digitorum longus muscle, we used immunohistochemistry method. The changes in muscle weight and fiber size were evaluated during 30-day

denervation. The cross-sectional area of muscle fibers was assessed by computerized image analysis.

Materials and Methods

Animals

Twenty-seven male Wistar rats, 2.5-3 months old (body weight 300±20 g), were used. Over the period of the study, all animals were provided with standard laboratory food and water *ad libitum*.

Surgical Procedures

Prior to the experiments, the animals were anesthetized with an intraperitoneal injection of ketaminhidroxichloride (Ketamine, Parke-Davis, Milan, Italy), in a dose of 25 mg/kg. Denervation of the extensor digitorum longus muscle was performed by unilateral right sciatic nerve transection proximally to its branching. Removing a 0.5 cm segment of the nerve and placing the ends into nearby muscles prevented reinnervation. The overlying muscles and skin were then sutured. The animals were sacrificed by cervical hyperextension in groups of six rats, 6, 12, and 30 days after denervation. Non-operated animals in groups of 3 served as a control. Experimental muscles were carefully freed from the surrounding tissue, removed, weighted, frozen in liquid nitrogen, and stored at -80°C until further analysis.

Immunohistochemistry

Monoclonal antibodies specific for fiber type I (BF-F8), IIA (SC-71), IIB (BF-F3) and type IIX (BF-35) (17,18) were used in staining a serial of 10 µm transverse cryosections for slow and fast myosin heavy chain isoforms. Prof. Stefano Schiaffino, Department of Biomedical Sciences, Padua, Italy donated the antibodies. Slides were incubated with monoclonal antibodies (1:1000) in phosphate-buffered saline (PBS) at room temperature for 30 minutes. After three wash steps in PBS, slides were incubated at room temperature for 30 min with peroxidase-labeled rabbit anti-mouse IgG (Dako A/S, Copenhagen, Denmark) in PBS (1:40). After being washed twice in PBS, the slides were incubated in 50 mL of diaminobenzidine (DAB) solution (0.5 mol/L Tris HCl, pH 7.6, 15 mg of DAB, 100 µL of H_2O_2, 25 mg of imidazole) at room temperature for 20 min. Finally, the slides were dried and mounted in Canada balsam.

Fiber Typing and Morphometry

The fiber type frequencies and cross-sectional areas were analyzed by the computer program for quantitative analysis "SFORM" (VAMS, Zagreb, Croatia). One hundred fibers of each type were measured by moving a pen along the circumference of the fibers. Mean fiber size with standard deviation (SD) was calculated. Control groups were used for comparison with denervated muscles. Statistical evaluations were performed by Student t-test at $p<0.001$ level of significance.

Results

Muscle Mass

As a consequence of denervation, extensor digitorum longus muscle underwent atrophy, as evidenced by the decreased muscle mass, 6, 12, and 30 days after denervation (Fig. 1). Extensor digitorum longus muscle lost 23% of its muscle mass at control weight as early as 6 days afterdenervation. Muscle mass continued to decrease and one month after the denervation, it was reduced to only 44% of the control muscle mass.

Fiber Types following Denervation

Control extensor digitorum longus muscle was composed of 41% of type IIB fibers, 32% of type IIA, 23% of type IIX, and only 4% of fibers type I (Fig. 2). All fast fiber types were affected on 6th day of denervation ($p<0.001$ only for IIA and IIB fiber types). After 30 days of denervation, the number of type IIB and IIX fibers decreased. The percentage of type IIB fibers was reduced from 41% to 36% ($p<0.001$). The percentage ofIIX type of fibers was reduced even more, and after 30 days, decreased significantly from 23% to 13% ($p<0.001$). The percentage of fibers type IIA and type I increased from 32% to 42% and from 4% to 9%, respectively ($p<0.001$).

Fiber Size following Denervation

Morphometrical analysis performed on cross sectional area of particular muscle fiber type in control extensor digitorum longus muscle showed that fibers of type IIB were the largest, with the mean fiber size of 4,913.4±1,123.0 µm^2. Type IIX and IIA muscle fibers had a mean fiber size of 2,391.0±461.0 µm^2 and 1,841.6±447.0 µm^2, respectively. Finally, type I fibers had the smallest fiber size of 1,586.1±427.0 µm^2 (Fig. 3).

Six days after denervation, the cross sectional area of type IIB and IIX fibers was significantly reduced ($p<0.001$). One month later, the reduction of the fibre size of type IIX and IIB reached 75% and 72% of the control, respectively (Fig. 3). The mean fiber size of type IIB fibers was 1,373.8 ±413.0 µm^2 ($p<0.001$), whereas that of the type IIX fibers was 606.1±224.0 µm^2 ($p<0.001$). Six days after denervation, the atrophy of type IIA fibers was also statistically significant

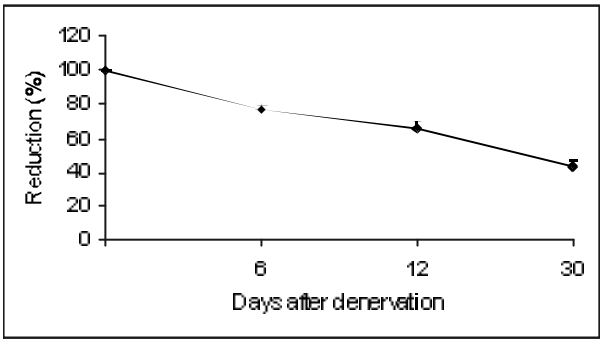

Figure 1. Reduction in rat extensor digitorum longus muscle mass 6, 12, and 30 days after denervation. The mass of denervated muscles was compared with a control muscles and expressed as a percentage of a control muscle. Each point represents the mean value of 6 muscles ± SD.

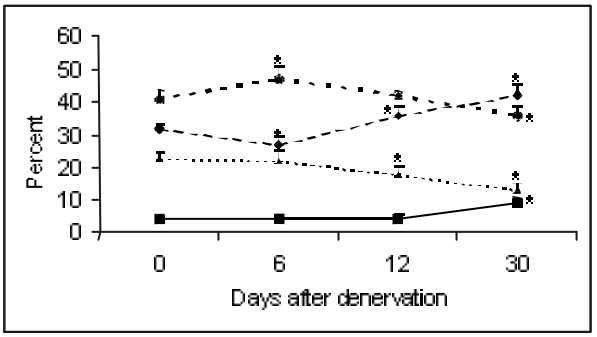

Figure 2. Changes in the fiber type content of adult extensor digitorum longus muscle 6, 12, and 30 days after denervation. Muscle fiber types are: square – type I, rhomb – type IIA, triangle – type IIX, and circle – type IIB. Each point represents mean value ± SD. All experimental groups were compared with the control group and the asterisk indicates statistical significance at $p<0.001$ level in comparison with control.

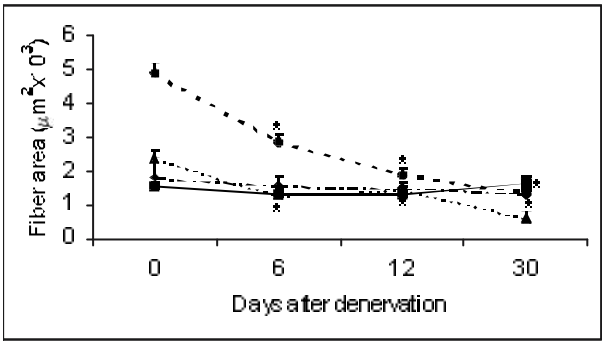

Figure 3. Changes in the cross-sectional area of adult extensor digitorum longus muscle 6, 12 and 30 days of denervation. Muscle fiber types are: square-type I, rhomb-type IIA, triangle-type IIX, and circle-type IIB. Each point represents mean value ± SD. All experimental groups were compared with the control group and the asterisk signindicates statistical significance at $p<0.001$ level in comparison with control.

($p<0.001$). After 30 days, the size reduction of type IIA fibers progressed, reaching the mean fiber size of 1,321.4±383.0 μm² ($p<0.001$). Type I fibers showed a reduction in fiber size in the first 12 days ($p<0.001$) but later on increased in size, reaching the control mean fiber size of 1,692.8±578.0 μm² ($p<0.001$).

Discussion

We demonstrated progressive atrophy, which was the most pronounced in type IIB and IIX muscle fibers, and changes in fiber type composition in rat extensor digitorum longus muscle one month after denervation. Our results suggest that all fast fibers are not equally dependent on neural influences. Previous studies performed on extensor digitorum longus muscle have shown either reduction in fiber size of muscle fibers type IIB only (11) or muscle atrophy of type IIA and IIB fibers, with type I fibers showing no significant alteration in cross- sectional area (12).

On the basis of specific enzymes in anaerobic or aerobic-oxidative metabolism, muscle fibers can be classified as either oxidative (type I) or glycolytic (type II) (16). We have shown that type I fibers increased only slightly in fiber size and seemed to be the most resistant fiber type to the loss of innervation. This is an interesting finding because, among type II fibers, the least susceptible for denervation are type IIA muscle fibers. These are considered fast type of fibers, which resemble the most to the oxidative type of fibers. In general, our results suggest that denervation causes changes in the fiber size of all types of muscle fibers, but predominantly of type II fibers, which seem to be more dependent on neural influences than type I fibers. Metabolic pathway of muscle fibers could be an important factor in response to denervation, since the oxidative type of fibers are the most resistant to a lack of innervation.

Muscle denervation eliminates both trophic and activity-related influences and thus triggers many changes in myosin heavy chain isoform expression (2). Our data indicate that fast-twitch extensor digitorum longus muscle responds to altered neuromuscular activity very rapidly by change in the muscle phenotype (Fig. 2). The alterations that ultimately lead to the changes in the level of individual myosin heavy chain isoform expression suggest that the process of denervation can rapidly shift the myosin heavy chain profile towards expressing slower isoforms, while decreasing the expression of the faster isoforms (IIB and IIX).

Muscle denervation inhibited the expression of IIB myosin heavy chain isoforms significantly. The reduction of the IIB fiber phenotype is not easy to explain, considering the fact that IIB mRNA continues to accumulate even without the continual innervation in newborn rat hindlimb muscles (19). On the other hand, the reduction of IIB myosin heavy chain isoform due to denervation was observed at the mRNA level in another fast muscle, the anterior tibial muscle (14). Our study also showed a drastic down-regulation in the expression of IIB myosin heavy chain in myotonic muscles (20-22), in chronic low-frequency stimulation of rat fast muscle (23), in exercise training (24), and in mechanical overloading (25). In our study, the expression of IIX myosin heavy chain was significantly diminished, which is in disagreement with the demonstration of increased proportion of IIX myosin heavy chain in 14-day denervated extensor digitorum longus muscle (15), but consistent with a study that revealed the reduction of IIX myosin heavy chain mRNA in anterior tibial muscle (14). Interestingly, in both slow (i.e., soleus muscle) and fast (extensor digitorum longus) muscles, the lack of innervation increased the expression of IIA myosin heavy chain, suggesting that IIA myosin heavy chain expression in the absence of the nerve represents a default program (2). The proportion of type I muscle fibers increased significantly, too. This suggests that the "fast" motor nerve suppresses the expression of the type I myosin heavy chain.

Our findings may have important clinical relevance. Demonstration of changes in the early phases of denervation indicates that electrical stimulation of denervated muscles by correct pattern of frequency (26) could prevent progressive atrophy and changes in muscle phenotype caused by a lack of neural control.

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