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The Effect of a Unilateral Muscle Transplantation on the Muscle Fiber Type and the MyHC Isoform Content in Unoperated Hind Limb Slow and Fast Muscles of the Inbred Lewis Rats

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Summary

To reveal the effect of foreign innervation and altered thyroid status on fiber type composition and the myosin heavy chain (MyHC) isoform expression in the rat slow soleus (SOL) and fast extensor digitorum longus (EDL) muscles, a method of heterochronous isotransplantation was developed. In this experimental procedure, the SOL or EDL muscles of young inbred Lewis rats are grafted either into the host EDL or SOL muscles of adult rats of the same strain with normal or experimentally altered thyroid status. To estimate the extent of fiber type transitions in the transplanted muscles, the SOL and EDL muscle from the unoperated leg and unoperated muscles from the operated leg could be legitimately used as controls, but only when the experimental procedure itself does not affect these muscles. To verify this assumption, we have compared the fiber type composition and the MyHC isoform content of unoperated contralateral SOL and EDL muscles and ipsilateral unoperated SOL muscle of experimental rats after unilateral isotransplantation into the host EDL muscle with corresponding muscles of the naive rats of the same age and strain. We provide compelling evidence that the unilateral heterochronous isotransplantation has no significant effect on the fiber type composition and the MyHC isoform content of unoperated muscles of experimental animals. Hence, these muscles can be used as controls in our grafting experiments.

Key words

Rat hind limb muscles • Muscle fiber phenotype • Stereology • Immunocytochemistry • Gel electrophoresis • Influence of surgery • Muscle transplantation

Introduction

The analysis of the influence of intrinsic (genetic) and extrinsic factors governing the diversification of muscle fibers during normal and pathological development involves the study of muscle phenotype in regenerated grafted muscles. For this purpose, our method of heterochronous isotransplantation

is used (Jirmanová and Soukup 1995). In this experimental procedure, the soleus (SOL) or extensor digitorum longus (EDL) muscles of young inbred Lewis rats are intramuscularly grafted into the host EDL or SOL muscle of adult animals of the same strain. To estimate the extent of fiber type transitions in the transplanted muscles, the unoperated contralateral and ipsilateral SOL or EDL muscles from experimental animals could be used as control muscles to the experimental (transplanted and host) muscles, but only when the experimental procedure itself does not affect these muscles. Although the experimental animals recover soon after the transplantation and their mobility seems to be unaffected, it is possible that hind limb muscles could be affected by the experimental procedure. Since the host muscle of the experimental animals is loaded with a foreign muscle, the operated animals may not use the operated limb to the same extent as the contralateral, unoperated one. Therefore, it might be expected that the contralateral limb had been overloaded. It has previously been demonstrated that loading and unloading induces the fiber type transitions, especially in the slow muscles (e.g. Asmussen and Soukup 1991, Pette and Staron 1997, Caiozzo *et al.* 1998, Goldspink 1999, Fitts *et al.* 2000, Talmadge 2000, Baldwin and Haddad 2001). Therefore, the changes in fiber type composition and the myosin heavy chain (MyHC) isoform content of control muscles not containing the graft might also occur. In order to find out whether the control muscles from the experimental rats were affected by the unilateral muscle transplantation, we determined the fiber type composition and the MyHC isoform content in the unoperated (contralateral and ipsilateral) SOL and EDL muscles of the experimental rats and compared it with corresponding normal muscles of the naive rats.

Methods

Muscle samples

In this study, we have analyzed muscle samples from female inbred rats of the Lewis strain, obtained from an authorized laboratory of the rat-breeding unit of the Institute of Physiology (Accreditation number 1020/491/A/00). The maintenance and handling of experimental animals followed the EU Council Directive (86/609EEC) and the investigation was approved by the Expert Committee of the Physiological Institute of the Academy of Sciences, Prague, Czech Repuiblic. Rats 4- to 10-month-old, (mean age 7.0±2.9 months), were divided into a "naive" normal (not operated) and an experimental (operated) group; in this latter group, the heterochronous isotransplantation of the SOL or EDL muscles into the host EDL muscle was performed (for detailed description of the grafting procedure see Jirmanová and Soukup 1995)**.** From the normal group, the SOL and EDL muscles from the left (SOLsin, EDLsin) and right (SOLdx, EDLdx) hind limb were excised. From the experimental animals, the SOL and EDL muscles from the unoperated, contralateral limb (SOLcl, EDLcl) and the SOL from the operated, ipsilateral limb (SOLil) were analyzed in this study. The ipsilateral EDL (EDLil) was not available for the present study, as it served as the host muscle for the graft.

After the rats had been anesthetized and euthanized by an intraperitoneal injection of Nembutal (sodium pentobarbital 40 mg/kg), the muscles were excised and frozen in liquid nitrogen. Serial cryosections of 10 μm were prepared from the central part of each muscle on a cryocut (Leica 3000), while the upper and the lower parts of the muscle were processed for sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

Antibodies

To identify the MyHC isoforms in the muscle fibers, different mouse monoclonal antibodies (mAbs) specific to MyHC isoforms were used: aS (MyHC-slow) and aF (MyHC-fast), both provided by Biotrend or Medac/Novocastra (Germany). To further detect slow and fast MyHC isoforms mAbs BA-D5 (MyHC-1), SC-71 (MyHC-2a), BF-35 (all isoforms except MyHC-2x/d) and BF-F3 (MyHC-2b) were used (Schiaffino *et al.* 1986).

mATPase histochemistry and immunocytochemistry

The activity of myofibrillar adenosine triphosphatase (mATPase) activity (E.C.3.6.1.3) was demonstrated after alkaline (pH 10.3) and acid (pH 4.5 and 4.3) preincubations (Guth and Samaha 1970). To reveal the MyHC isoform expression in muscle fibers, the muscle cross-sections were incubated with primary antibody in a humidified box at 4 ºC overnight. Primary antibody binding was revealed with a standard indirect peroxidase-antiperoxidase (PAP) technique (Sternberger 1986) using the PAP products (Dakopatts, Copenhagen, Denmark) or with a donkey secondary antibody conjugated with horse-radish peroxidase (Jackson Immunoresearch Laboratories, USA).

SDS-PAGE

The muscle extracts for SDS-PAGE were prepared according to two protocols, which yielded comparable results. The muscle samples were either minced on ice and washed with five volumes of 20 mM NaCl, 5 mM sodium phosphate and 1 mM EGTA (pH 6.5). Myosin was extracted with 100 mM sodium phosphate, 5 mM EGTA and 1 mM dithiothreitol (pH 8.5) and the supernatant containing myosin was diluted with one volume of glycerol (87 %) and stored at –20 ºC. In the second protocol, the muscle samples (approximately 30 µg), were cut by scissors in an ice-cold homogenization buffer (1 mg of muscle per 40 µl of homogenization buffer: 5 M urea; 2 M thiourea; 10 mM pyrophosphate tetrasodium decahydrate; 0.13 % 2-mercaptoethanol). The minced muscles were subsequently homogenized in a glass tissue grinder, incubated for 10 min at 95 ºC and 4 µl of the homogenate was loaded onto the gel. MyHC isoforms were separated by SDS-PAGE (Talmadge and Roy 1993), carried out at constant voltage (70 V) for 30 h at 4 ºC. After the MyHC isoform separation, the gels were silver-stained (Blum *et al*. 1987).

Quantitative analyssis

The numerical (N) and areal (A) proportions (%) of muscle fiber types determined according to the mATPase reaction and the MyHC isoform content were assessed by the 2-D stereological method using the principles of unbiased counting frame and point counting (Zachařová and Kubínová 1995). A quantitative morphometry was performed using the stereological C.A.S.T. Grid System (Olympus, Albertslund, Denmark). The data were expressed as means ± S.D. and the significance of differences was evaluated by Student's t-test. The individual MyHC isoforms, separated by SDS-PAGE were densitometrically evaluated using AIDA 3.28 computer program (Advanced Image Data Analyzer, Germany).

Results and Discussion

Comparative analysis revealed no significant differences in the fiber type and the MyHC isoform content among any of the four SOL muscles analyzed. These comparisons included those between the SOLdx and SOLsin muscles from the normal rats and between the SOLcl and SOLil from the experimental rats. Furthermore, there were also no significant differences found among the SOL muscles from the normal and the experimental rats (Table 1 SOL). Similarly, the fiber type and the MyHC isoform content did not significantly differ either between the EDLdx and EDLsin muscles of the normal rats or among the EDL muscles of the normal and the EDLcl of the experimental rats (Table 1 EDL). Since the EDLil of the experimental animals was used as the host muscle for the graft, we could compare both SOL muscles (SOLcl and SOLil), but only EDLcl muscle of the experimental animals with the corresponding muscles of the normal rats.

Our results effectively demonstrated that heterochronous isotransplantation of muscles in our experiments had no significant influence on the fiber type composition and the MyHC isoform content of SOLcl, SOLil and EDLcl muscles from experimental rats. Hence, these muscles can be used as reliable controls to the regenerated muscle grafts in these studies, allowing a determination of the effect of genetic, neuronal, mechanical or hormonal factors on the regulation of the muscle phenotype using the model of heterochronous isotransplantation. In the first set of these experiments, we are studying the combined effect of "fast" innervation and the genetic properties, derived from "slow" or "fast" muscle precursors (satellite cells, derived either from slow SOL or fast EDL muscles) in rats with normal or experimentally altered thyroid status (Soukup *et al.* 2001, 2003, Hudecová *et al.* 2004, Vadászová *et al.* 2004a,b, for review see Soukup and Jirmanová 2000).

In the present study, the muscles of only 4- to 10-month-old rats have been compared. As the experimental animals were about 2-month-old at the time of the isotransplantation and the graft regeneration was allowed to proceed for another 2 to 8 months in this group, the youngest experimental rats, available for our comparative study with the naive rats were 4 months old. We found that the fiber type composition and the MyHC isoform content of the SOL and EDL muscles had not substantially changed after the fourth month of age in the normal and in the experimental rats. This indicates that the muscles are already mature at the age of four months and that the age (between 4 and 10 months) of individual animals had no effect on the fiber type and the MyHC composition of the analyzed muscles.

Table 1 SOL. Fiber type proportions of the right and left soleus muscles from normal (SOLdx, SOLsin) and experimental (SOLcl, SOLil) in rats 4- to 10-month-old. The results are expressed as numerical (N%) and areal (A%) percentages of individual fiber types; slow or type 1 fibers were demonstrated by the mATPase reaction at pH 4.5 and immunohistochemically using anti slow (aS) and anti MyHC-1 (BA-D5) mAbs, type 2A fibers (expressing MyHC-2a) were revealed by anti fast (aF) and anti MyHC-2a (SC-71) mAbs. MyHC-1 isoform content was revealed by SDS-PAGE (SDS1). Note that there are no differences neither between the SOL muscles of the normal rats nor between the SOL muscles of the experimental rats and among the SOL muscles of the normal and the experimental animals as well. Note that some muscle fibers co-expressed MHC-1 and MHC-2a as indicated by N% values that exceed 100 % (in N% 0.2 – 1.7). n = number of animals.

	ATPase (N)	**ATPase (A)**	**BA-D5 (N)**	**BA-D5 (A)**	**aS (N)**	**aS (A)**	**SC-71 (N)**	**SC-71 (A)**	**aF (N)**	**aF (A)**	**SDS 1**
SOL dx	99.2 ± 1.7	99.3 ± 1.5	98.9 ± 2.3	99.1 ± 2.0	100.0 ± 0.0	100.0 ± 0.0	1.4 ± 2.4	1.1 ± 2.1	1.7 ± 2.5	1.4 ± 2.1	99.1 ± 1.9
n	6	6	5	5	4	4	5	5	5	5	5
SOL sin	98.4 ± 2.7	98.8 ± 2.3	98.5 ± 2.4	98.6 ± 2.2	98.0 ± 3.2	98.4 ± 2.6	2.3 ± 3.1	2.1 ± 2.9	3.7 ± 5.6	2.7 ± 4.3	97.6 ± 4.2
n	5	5	4	4	4	4	4	4	4	4	5
SOL cl	99.2 ± 1.7	99.4 ± 1.2	98.7 ± 2.5	99.0 ± 2.0	99.5 ± 1.6	99.7 ± 1.0	1.8 ± 2.9	1.2 ± 1.7	0.8 ± 1.6	0.7 ± 1.3	97.6 ± 3.6
n	13	13	4	4	11	11	4	4	11	11	6
SOL il	98.6 ± 2.2	99.1 ± 1.4	99.0 ± 2.2	99.3 ± 1.5	98.8 ± 2.6	99.1 ± 1.7	2.1 ± 2.3	1.5 ± 1.8	2.3 ± 3.8	1.8 ± 3.2	97.8 ± 2.1
n	14	14	6	6	10	10	7	7	10	10	6

Table 1 EDL. The mean numerical (N%) and areal (A%) percentages of fiber types and MyHC isoform content in the EDL muscles of normal and experimental 4- to 10-month-old rats. Fiber types were determined according to the mATPase reaction, MyHC isoforms were determined immunocytochemically using anti slow (aS) and anti fast (aF) MyHC mAbs or specific mAbs recognizing MyHC-1 (BA-D5), -2a (SC-71), -2x/d (BF-35 negative marker) and -2b (BF-F3) MyHC isoforms and after SDS-PAGE (SDS1, SDS2a, SDS2x/d, SDS2b) analysis. SFcoex and Abcoex indicate coexpression as revealed by anti slow and anti fast or with all four specific mAbs, respectively. n= number of animals.

N%	**ATP 1**	**ATP 2A**	**ATP 2B**	**BA-D5**	**SC-71**	**BF-35**	**BF-F3**	**Ab coex**	**aS**	**aF**	**SF coex**
EDL dx	5.8 ± 1.3	17.0 ± 3.7	77.3 ± 3.5	5.3 ± 0.8	25.0 ± 4.6	34.3 ± 9.9	42.1 ± 7.3	107.6 ± 8.4	5.9 ± 1.8	95.7 ± 1.3	101.6 ± 0.9
n	6	6	6	6	7	6	6	6	7	7	7
EDL sin	5.9 ± 0.7	17.2 ± 2.9	76.8 ± 3.3	6.3 ± 1.4	21.4 ± 2.9	36.6 ± 8.1	39.8 ± 7.7	104.9 ± 9.4	6.7 ± 1.2	94.8 ± 1.2	101.5 ± 1.1
n	6	6	6	6	7	6	6	6	7	7	7
A%	**ATP 1**	**ATP 2A**	**ATP 2B**	**BA-D5**	**SC-71**	**BF-35**	**BF-F3**	**SDS 1**	**SDS 2a**	**SDS 2x**	**SDS 2b**
EDL dx	2.5 ± 0.8	9.1 ± 2.4	88.4 ± 2.8	2.5 ± 0.5	11.0 ± 3.1	32.6 ± 12.2	52.2 ± 6.9	3.3 ± 1.6	13.7 ± 3.1	35.0 ± 4.6	48.1 ± 7.4
n	6	6	6	6	7	6	6	9	9	9	9
EDL sin	2.4 ± 0.4	8.9 ± 1.2	88.8 ± 1.5	2.6 ± 0.7	10.0 ± 2.0	33.6 ± 9.5	52.0 ± 8.2	4.3 ± 1.5	15.4 ± 4.1	35.9 ± 3.2	46.0 ± 5.8
n	6	6	6	6	7	6	6	9	9	9	9
N%	**ATP 1**	**ATP 2A**	**ATP 2B**	**BA-D5**	**SC-71**	**BF-35**	**BF-F3**	**Ab coex**	**aS**	**aF**	**SF coex**
EDL cl	6.0 ± 1.3	17.8 ± 3.4	76.2 ± 3.6	6.1 ± 2.6	23.4 ± 5.9	32.6 ± 6.1	44.5 ± 4.4	104.9 ± 6.9	5.4 ± 1.5	95.4 ± 1.9	100.8 ± 0.5
n	8	5	5	6	6	6	5	5	4	4	4
A%	**ATP 1**	**ATP 2A**	**ATP 2B**	**BA-D5**	**SC-71**	**BF-35**	**BF-F3**	**SDS 1**	**SDS 2a**	**SDS 2x**	**SDS 2b**
EDL cl	± 0.5	2.0 ± 2.9	7.690.4 ± 3.3	1.8 ± 0.5	10.0 ± 2.5	34.4 ± 6.0	49.4 ± 10.1	4.4 ± 2.1	17.5 ± 4.7	38.3 ± 7.4	39.8 ± 9.1
n	3	3	3	4	4	4	4	6	6	6	6

The fiber type composition and the MyHC isoform content of SOL and EDL muscles of the normal female Lewis strain rats have already been determined in our previous study, but only the muscles of 4- to 6-month-old rats were analyzed using the mATPase reaction and the SDS-PAGE technique (Soukup *et al.* 2002). In the present study, there was not only a wider age range of groups included, but the fiber type composition and the MyHC isoform content have also been accomplished with the immunohistochemistry using a battery of mAbs specific to MyHC isoforms and with a semiquantitative densitometrical evaluation of the electrophoretically separated MyHC isoforms. Contrary to the mATPase reaction, the methods included in the current study enable the demonstration of all fast MyHC isoforms (MyHC-2a, -2x/d and -2b), which is especially important in the case of the fast EDL muscle.

As the heterochronous isotransplantation had no effect on the fiber type and the MyHC isoform composition, it can be concluded that the experimental animals used and loaded both legs in a similar fashion. This is especially important in the case of the slow SOL muscle, in which the unloading or reduced weight-bearing activity lead to transition from slow to fast fiber type, whereas a chronic or an intermittent overloading leads to changes in the opposite direction (for review see Fitts *et al.* 2000, Talmadge 2000, Baldwin and Haddad 2001). Furthermore, the percentage of hybrid fibers, which are expressing more than one MyHC isoform and are normally more numerous during the fiber type transition (for review see Schiaffino and Reggiani 1996, Stephenson 2001, Pette 2002), was not prominent in the analyzed unoperated muscles of the experimental rats and was comparable to that of the normal animals. This is a further indication that the experimental procedure has not induced the fiber type transition in these muscles.

Although many muscle diseases do not belong to diseases characterized by current high scientific or medical concern (such as cancer, cardiovascular or infectious diseases), severe muscular diseases including Duchenne or limb girdle dystrophies have devastating effects on patients. Not surprisingly, these diseases often affect young children and result in an extreme deterioration of their well-being, significantly shortening their lives. Furthermore, muscle diseases connected with the elderly, have become more widespread as life expectancy continues to increase in many countries. Therefore, we hope that our research might contribute to a better understanding and perhaps to improved treatment of various pathological muscle alterations.

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References

ASMUSSEN G, SOUKUP T: Arrest of developmental conversion of type II to type I fibers after suspension hypokinesia. *Histochem J* **23**: 312-322, 1991.

BALDWIN K, HADDAD F: Plasticity in skeletal, cardiac, and smooth muscle. Effects of different activity and inactivity paradigms on myosin heavy chain gene expression in striated muscles. *J Appl Physiol* **90**: 345-357, 2001.

BLUM H, BEIER H, GROSS HJ: Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. *Electrophoresis* **8**: 93-99, 1987.

CAIOZZO VJ, BAKER MJ, BALDWIN KM: Novel transitions in MHC isoforms: separate and combined effects of thyroid hormone and mechanical unloading. *J Appl Physiol* **85**: 2237-2248, 1998.

FITTS RH, RILEY DR, WILDRICK JJ: Microgravity and skeletal muscle. *J Appl Physiol* **89**: 823-839, 2000.

GOLDSPINK G: Changes in muscle mass and phenotype and the expression of autocrine and systemic growth factors by muscle in response to stretch and overload. *J Anat* **194**: 323-334, 1999.

GUTH L, SAMAHA FJ: Procedure for the histochemical demonstration of actomyosin ATPase. *Exp Neurol* **28**: 365-367, 1970.

HUDECOVÁ S, VADÁSZOVÁ A, SOUKUP T, KRIŽANOVÁ O: Effect of thyroid hormones on the gene expression of calcium transport systems in rat muscle. *Life Sci* **75**: 923-931, 2004.

JIRMANOVÁ I, SOUKUP T: Critical period in muscle spindle regeneration in grafts of developing rat muscle. *Anat Embryol* **192**: 283-291, 1995.

PETTE, D. The adaptive potential of skeletal muscle fibers. *Can J Appl Physiol* **27**: 423-448, 2002.

PETTE D, STARON RS: Mammalian skeletal muscle fibre type transitions. *Int Rev Cytol* **170**: 143-223, 1997.

SCHIAFFINO S, SAGGIN L, VIEL A, AUSONI S, SARTORE S, GORZA L.: Muscle fiber types identified by monoclonal antibodies to myosin heavy chains. In: *Biochemical Aspects of Physical Exercise.* G BENZI, L PACKER, N SILIPRANDI (eds), Elsevier, Amsterdam, 1986, pp 27-34.

SCHIAFFINO S, REGGIANI C: Molecular diversity of myofibrillar proteins: gene regulation and functional significance. *Physiol Rev* **76**: 371-423, 1996.

SOUKUP T, JIRMANOVÁ I: Regulation of myosin expression in developing and regenerating extrafusal and intrafusal muscle fibres with special emphasis on the role of thyroid hormones. *Physiol Res* **49**: 617-633, 2000.

SOUKUP T, ZACHAŘOVÁ G, SMERDU V, JIRMANOVÁ I: Body, heart, thyroid gland and skeletal muscle weight changes in rats with altered thyroid status. *Physiol Res* **50**: 619-626, 2001.

SOUKUP T, ZACHAŘOVÁ G, SMERDU V: Fibre type composition of soleus and extensor digitorum longus muscles in normal female inbred Lewis rats. *Acta Histochem* **104**: 399-405, 2002.

SOUKUP T, ZACHAŘOVÁ G, VADÁSZOVÁ A, JIRMANOVÁ I, SMERDU V, ASMUSSEN G, KRIŽANOVÁ O, NOVOTOVÁ M: Contribution of innervation and thyroid hormones to the differentiation of muscle fibre phenotype. *Physiol Res*, **52**: 19P, 2003.

STEPHENSON GMM: Hybrid skeletal muscle fibres: a rare or common phenomenon? *Clin Exp Pharmacol Physiol* **28**: 692-702, 2001.

STERNBERGER LA: *Immunocytochemistry*. Wiley, New York, 1986.

TALMADGE RJ, ROY RR: Electrophoretic separation of the rat skeletal muscle myosin heavy-chain isoforms. *J Appl Physiol* **75**: 2337-2340, 1993.

TALMADGE RL: Myosin heavy chain isoform expression following reduced neuromuscular activity: potential regulatory mechanisms. *Muscle Nerve* **23**: 661-679, 2000.

VADÁSZOVÁ A, ZACHAŘOVÁ G, MACHÁČOVÁ K, JIRMANOVÁ I, SOUKUP T: Influence of thyroid status on the differentiation of slow and fast rat muscle phenotypes. *Physiol Res* **53** (Suppl 1): S57-S61, 2004a.

VADÁSZOVÁ A, ZACHAŘOVÁ G, ASMUSSEN G, HUDECOVÁ S, KRIŽANOVÁ O, SOUKUP T: Thyroid hormones regulate differently muscle MHC isoforms and their mRNA levels. *Physiol Res* **53**: 42P, 2004b.

ZACHAŘOVÁ G, KUBÍNOVÁ L: Stereological methods based on point counting and unbiased counting frames for two-dimensional measurements in muscles: comparison with manual and image analysis methods. *J Muscle Res Cell Motil* **16**: 295-302, 1995.

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