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## Variability in fibre properties in paralysed human quadriceps muscles and effects of training

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**Abstract** A spinal cord injury usually leads to an increase in contractile speed and fatigability of the paralysed quadriceps muscles, which is probably due to an increased expression of fast myosin heavy chain (MHC) isoforms and reduced oxidative capacity. Sometimes, however, fatigue resistance is maintained in these muscles and also contractile speed is slower than expected. To obtain a better understanding of the diversity of these quadriceps muscles and to determine the effects of training on characteristics of paralysed muscles, fibre characteristics and whole muscle function were assessed in six subjects with spinal cord lesions before and after a 12-week period of daily low-frequency electrical stimulation. Relatively high levels of MHC type I were found in three subjects and this corresponded with a high degree of fusion in 10-Hz force responses ( $r=0.88$ ). Fatigability was related to the activity of succinate dehydrogenase

(SDH) ( $r=0.79$ ). Furthermore, some differentiation between fibre types in terms of metabolic properties were present, with type I fibres expressing the highest levels of SDH and lowest levels of  $\alpha$ -glycerophosphate dehydrogenase. After training, SDH activity increased by  $76\pm 26\%$  but fibre diameter and MHC expression remained unchanged. The results indicate that expression of contractile proteins and metabolic properties seem to underlie the relatively normal functional muscle characteristics observed in some paralysed muscles. Furthermore, training-induced changes in fatigue resistance seem to arise, in part, from an improved oxidative capacity.

**Keywords** Contractile properties · Electrical stimulation · Fatigue · Metabolism · Muscle fibre type · Myosin heavy chain · Paraplegia · Vastus lateralis

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### Introduction

There is general consensus among researchers that expression of contractile proteins in skeletal muscle is affected by neural influences, in addition to hormonal factors and the origin of myogenic cells and primary/secondary fibre generations [23]. Furthermore, expression of myosin heavy chain (MHC) isoforms seems a major determinant of the contractile properties and fatigue resistance of skeletal muscle fibres [23]. In line with these ideas, it has been demonstrated that spinal cord injury (SCI) results in an increased expression of fast contractile proteins (MHC IIa and IIx) [1, 4] and an increased contractile speed [8, 20]. The low oxidative capacity [14, 21] accompanying the altered fibre type expression probably limits the fatigue resistance observed in these muscles [8, 20].

Despite these consistent findings that SCI leads to pronounced adaptations in the metabolic properties and phenotypic expression of the paralysed muscle fibres, we have recently observed unexpected slow contractile properties and high fatigue resistance in the quadriceps muscles of some individuals with long-term (>2 years)

**Table 1** Characteristics of subjects with spinal cord injury with respect to lesion, myosin heavy chain (MHC) expression and functional properties of the quadriceps muscle (FOA force oscillation amplitudes)

Subject	Lesion characteristics		MHC expression (%)			Quadriceps function	
	Level	Duration (years)	I	Ia	Iix	FOA	Fatigue resistance <sup>a</sup> (%)
1	C7	29	50	21	30	23	68
2	T7	13	35	27	38	40	41
3	T9	12	62	32	6	35	29
4	T5	2.5	1	12	87	83	24
5	C5	13	0	12	88	72	19
6	C5	20	0	33	67	114	28

<sup>a</sup> Fatigue resistance was defined as percentage of pre-fatigue force at 2 min of stimulation. See Materials and methods for further explanation and definitions

complete SCI [9], whilst others exhibited functional characteristics that are more typical for paralysed muscle [8]. The large variability in contractile properties was specifically represented by the degree of fusion at low-frequency stimulation (low and high fusion indicating fast and slow muscle, respectively). These results suggest relatively high levels of oxidative metabolism and MHC I expression in those subjects with functional characteristics, resembling healthy control subjects.

In addition, when the quadriceps muscles of SCI subjects were subjected to 12 weeks of training with low-frequency (10 Hz) electrical stimulation we observed increased muscle strength, contractile slowing, and increased fatigue resistance.

To improve our understanding of the underlying processes responsible for the variability in functional properties of paralysed muscles, investigation of histochemical and molecular properties of these muscles is needed. The purpose of this study was to assess the oxidative and glycolytic capacity, as well as the MHC expression, of vastus lateralis biopsy samples of paralysed quadriceps muscles that show different contractile speed and fatigue resistance. Secondly, this study aimed to investigate changes in these fibre properties as a result of training with electrical stimulation.

## Materials and methods

### Subjects

The present study included five males and one female (age: 29–56 years, height: 1.62–1.85 m, weight: 60–90 kg) with long-term (>2 years) SCI (Table 1). Lesion levels ranged between C5 and T9 and were all traumatic complete motor lesions classified as ASIA (American Spinal Injury Association [15]) A. Subjects had no pressure ulcers, nor severely reduced mobility in knee joints or previous bone fractures. All subjects showed presence of spinal reflexes of knee-extensor muscles (assessed by a physician), indicating absence of peripheral motor neuron lesion at the corresponding spinal level. Volunteers gave their written informed consent after procedures and involved risks with respect to the training and tests were explained. The Medical Ethical Committee of the University of Nijmegen approved this study.

### Quadriceps training

The quadriceps muscles were daily trained for a period of 12 weeks with either a portable custom-made electrical stimulation device (Technical services, Vrije University Amsterdam, The Netherlands) or a commercially available portable stimulator (Elpha 2000, Danica, Leusden, The Netherlands). Stimulation was with monophasic square-wave electrical pulses of 0.25 ms duration delivered via self-adhesive 50 mm×89 mm surface electrodes (Bioflex, PE 3590, Danica) placed over the proximal and distal part of the anterior thigh. Electrode placements were marked to ensure the same proportion of the muscle was stimulated throughout the training period (i.e. that part of the muscle from which biopsy samples were taken).

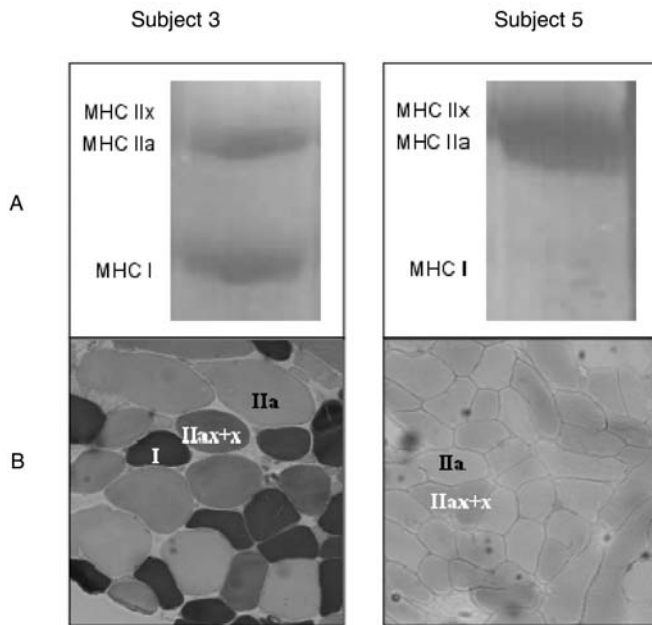
Muscles were trained with a low-frequency stimulation protocol, consisting of 40 min of repeated quadriceps activation with 10-Hz stimulation trains of 20 s duration, followed by 50 s rest. During a training session, the subjects sat in their wheelchairs with their knees flexed at an angle of approximately 90°, which was established with a goniometer (Electro Medico, Alphen a/d Rijn, The Netherlands). The lower limbs were fixed with a tight elastic strap placed around the ankles. An elastic strap was used instead of a rigid strap, to slacken sudden force increments, for instance during occasional muscle spasms. Consequently, some movement was allowed but this was only marginal (i.e. <5° change in knee angle), leading to near isometric muscle contractions.

### Muscle biopsy samples

Biopsy samples from each volunteer were taken before and after training from the middle proportion of the vastus lateralis muscle with a (Bergström) needle biopsy technique. Care was taken to obtain pre- and post-training biopsy samples from approximately the same region of the muscle. Samples for histochemistry were mounted in Tissue-TEK and frozen in isopentane, which was pre-cooled with liquid N<sub>2</sub>. A second sample was immediately frozen in liquid N<sub>2</sub> and freeze-dried for MHC analysis. All samples were stored at –80°C until analysed.

For histochemical analysis, serial sections (10 μm) were mounted on Vectabond-coated glass slides (four sections per slide). During each histochemical staining procedure, samples from all subjects were processed together. A standard sirius red staining [27], which can distinguish connective tissue from muscle tissue, was used to define the muscle fibre boundaries. These samples were used as a mask to identify each individual fibre during off-line histochemical analysis.

To characterize fibre types histochemically, muscle sections were stained for qualitative myofibrillar ATPase under acid (pH 4.7) and fixed alkaline (pH 10.4) preincubation conditions [3]. To investigate the metabolic profile of each fibre activities of SDH and α-glycerophosphate dehydrogenase (α-GPDH) were determined according to Martin et al. [13]. These enzymes may be used as indirect markers for the oxidative or glycolytic capacity of each fibre.



**Fig. 1** Typical examples of myosin heavy chain (MHC) distribution (A) and ATPase (pre-incubation at pH 4.7) staining (B) in two subjects with spinal cord injury. The *darkest-stained fibres* were defined as type I, *dark-grey fibres* as type IIax+x, and the *lightest-stained fibres* as type IIa. Note that there is a clear differentiation between fibre types in subject no. 3 (*left panels*), whereas this is much less obvious in subject no. 5 (*right panels*)

All analyses of histochemical staining were performed on an intelligent image analysis system (IBAS, Kontron Elektronik, Germany). Each sample was visualized by a microscope and a digital camera (Zeiss). From the sirius red-stained samples, masks were drawn for the selected fibres to be analysed and ATPase, SDH, and  $\alpha$ -GPDH stained samples were matched to these masks. Each fibre was identified by a number, which allowed summarizing all fibre characteristics (typing and oxidative/glycolytic activity) for each single fibre. The classification of fibre types as well as the SDH and  $\alpha$ -GPDH activity was based on the optical density measurements of each fibre. The optical density was linearly related to the enzymatic activity in the fibres. Descriptive statistics provided by the IBAS were used to identify fibre types and determine the metabolic properties of the different fibre types.

After the fibres were identified as type I or type II fibres, type II fibres were further classified as type IIa, IIax or IIx, according to their optical density, with type IIax representing hybrid fibres (Fig. 1). The lightest-stained type II fibres (ATPase, with pre-incubation at pH 4.7) were classified as type pure IIa (0% type IIx) and the darkest-stained type II fibres as pure type IIx (100% type IIx). The optical density values between these boundaries were distributed into categories of <25% type IIx (=type IIa), 25–75% type IIx (=type IIax), and >75% type IIx (=type IIx).

The majority of fibres were characterized as type IIa fibres and there were very few fibres that could be identified as pure type IIx. Therefore, fibres characterized as type IIax and IIx were taken together and are referred to as type IIax+x.

All subjects expressed type IIa fibres. Therefore, to demonstrate differences in SDH and  $\alpha$ -GPDH activity between individual fibre types, values of all fibres were expressed as a fraction of the mean optical density of histochemically characterized type IIa fibres. For comparisons of metabolic properties before and after training, total SDH and  $\alpha$ -GPDH activities were calculated by averaging optical densities of all fibres in each biopsy sample.

MHC composition was analysed with SDS-PAGE. Muscle homogenates were obtained by dissolving freeze-dried vastus

lateralis samples in boiling standard Lämmli sample buffer (2% SDS, 10% glycerol, 5% 2-mercaptoethanol, bromophenol blue in 0.125 M TRIS 0.002% HCL; pH ~6.8). Extracts from muscle homogenates were loaded onto SDS-PAGE gels for MHC separation. The separating and stacking gel contained 8% and 4% polyacrylamide, respectively. Gels were run for 24 h at 4°C, 350 V and subsequently silver stained. Three MHC isoforms could be identified (i.e. MHC I, IIa and IIx), and the relative content of each isoform within each sample was determined with integrated optical densitometry.

Because some muscle samples contained several obliquely cut fibres, standard calculations of fibre cross-sectional areas were not possible with measurements of fibre circumference (obtained from the masks). Alternatively, we measured the least diameter of fibres in each sample. Although changes in fibre diameter do not provide quantitative information about altered fibre cross-section it may be used as an indicator of qualitative adaptations in fibre morphology [16]. Therefore, it is assumed that an increase in fibre diameter would be a consequence of an increase in the number of contractile proteins and hence fibre hypertrophy.

#### Quadriceps dynamometry

The methods to determine contractile characteristics have been reported in detail elsewhere [8, 9]. Briefly, quadriceps muscles were electrically activated while subjects were seated and fixed on a specially designed chair with their lower limb attached to a force transducer and the knee at an angle of 100°. Isometric force responses were induced with surface electrical stimulation at different stimulation frequencies. Fatiguing contractions were generated using repeated 1-s bursts (30 Hz) every 2 s for 2 min; 30 Hz stimulation was chosen since this stresses the oxidative capacity of the muscle sufficiently without inducing high-frequency fatigue. Further, we have recently shown that with such a fatiguing protocol large differences can be demonstrated between muscles with different levels of oxidative metabolism [8].

The degree of fusion as a result of 10-Hz stimulation was determined by calculating the force oscillation amplitudes (FOA) relative to the mean force according to the method previously described [8]. Basically, larger relative force oscillation amplitudes indicate lower degree of fusion. Fatigue resistance was determined by expressing the force at the end of the 2-min fatiguing protocol relative to the pre-fatiguing force.

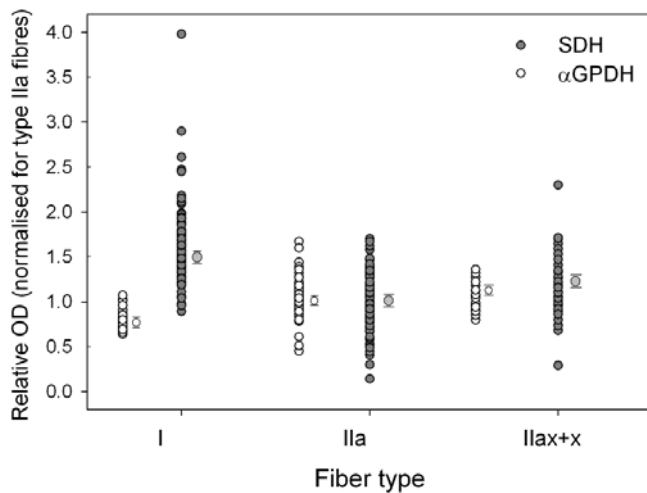
#### Statistical analysis

A non-parametric Mann-Whitney *U*-test was used to test for differences between characteristics of pre- and post-trained muscle samples. A 2-way analysis of variance was used to test for differences between fibre types and effects of training on oxidative and glycolytic capacities. Tukey post-hoc tests were performed in case of main effects of fibre types. Pearson correlation coefficients were calculated between muscle function and histochemistry/MHC content. All values are expressed as mean $\pm$ SE unless otherwise stated, and levels of  $P < 0.05$  were chosen as statistically significant.

## Results

### MHC expression

A substantial variation in MHC distribution could be observed among the individuals (Table 1). Whereas some subjects expressed predominantly MHC IIa and/or IIx, others expressed relatively high proportions of MHC I. This expression of MHC I, obtained from gel electrophoresis, correlated well with the proportions of histochem-



**Fig. 2** Activity of metabolic enzymes of different fibre types in non-trained vastus lateralis muscles. Type IIa (representing hybrid fibres; see Materials and methods) and IIx fibres were pooled. Data represent relative optical densities (ODs) (normalized for the mean value of that found in type IIa fibres) in fibres stained for succinate dehydrogenase (SDH; filled circles,  $n=606$ ) and  $\alpha$ -glycerophosphate dehydrogenase ( $\alpha$ -GPDH; open circles,  $n=365$ ). Symbols with error bars next to individual fibre data represent mean  $\pm$  SE. Fibres were obtained from all subjects (except subject no. 2; data for individual fibres could not be obtained from this subject)

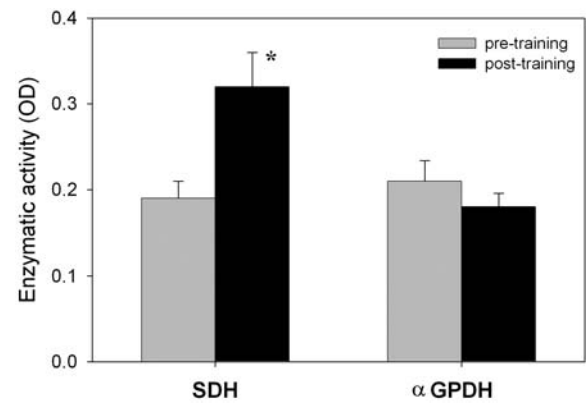
ically stained type I muscle fibres ( $r=0.87$ ,  $P<0.05$ ). Furthermore, the proportion of MHC I was closely related to the FOA ( $r=0.88$ ,  $P<0.05$ ) but non-significantly related to the fatigue resistance ( $r=0.59$ ,  $P>0.05$ ). As the proportion MHC I increases, FOA is reduced (the latter indicating a higher degree of fusion).

After training, the mean proportion of MHC I ( $26\pm 11\%$ ), IIa ( $25\pm 4\%$ ) and IIx ( $49\pm 9\%$ ) was not significantly different from pre-training values ( $24\pm 11\%$ ,  $23\pm 4\%$ ,  $53\pm 14\%$ , respectively).

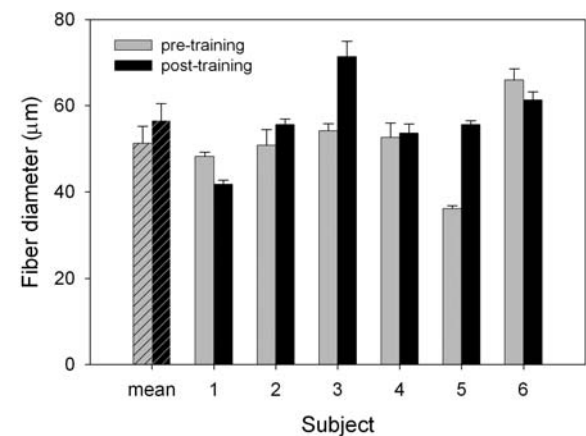
### Metabolic properties

Figure 2 presents SDH and  $\alpha$ -GPDH activities of all individual fibres obtained from muscle samples before training. SDH and  $\alpha$ -GPDH activities were significantly different between fibre types (of non-trained muscles) particularly in those subjects who expressed all three fibre types. When all fibres were pooled mean SDH activity was  $1.5\pm 0.02$  for type I, and  $1.18\pm 0.02$  for type IIax+x. Mean  $\alpha$ -GPDH activity was  $0.77\pm 0.01$  for type I, and  $1.01\pm 0.01$  for type IIax+x. Total SDH activity (average value of all fibres in one biopsy sample) was significantly related to the fatigue resistance found in the corresponding quadriceps muscle ( $r=0.81$ ,  $P<0.05$ ).

Twelve weeks of daily electrical stimulation training resulted in a significant increase by  $76\pm 26\%$  ( $P<0.05$ ) in total SDH activity in the trained vastus lateralis muscle (Fig. 3). This increase in SDH activity occurred in all different fibre types (data not shown). In addition, the



**Fig. 3** SDH and  $\alpha$ -GPDH activities before (grey bars) and after (black bars) training. Data represent mean group values, expressed as ODs. Error bars represent SE. \*Significantly different,  $P<0.05$



**Fig. 4** Muscle fibre diameters before (grey bars) and after (black bars) training. Group values (hatched bars) as well as individual data are presented. Error bars represent SE

activity of  $\alpha$ -GPDH was not significantly altered after training.

### Fibre diameter

Figure 4 presents muscle fibre diameters obtained from vastus lateralis samples before and after training. Before training, mean fibre diameter was  $51\pm 4$   $\mu\text{m}$ . Although some individuals showed a significant increase or decrease in fibre diameter, over all, no clear tendency for a change in fibre diameter could be observed after training (mean fibre diameter post-training:  $56\pm 4$   $\mu\text{m}$ ).

## Discussion

This study shows interesting data of variability in muscle fibre properties in individuals with SCI. Although some subjects showed expected predominance of MHC IIx, others still expressed fairly large proportions of MHC I.

This variation in muscle fibre composition as well as the metabolic properties seems related to the functional characteristics of these muscles. Finally, as a result of training with low-frequency electrical stimulation, oxidative capacity increased. However, training did not change fibre diameter or muscle fibre composition.

#### Untrained paralysed quadriceps muscles

Vastus lateralis muscles of non-disabled individuals usually contain approximately 50% type I fibres [24]. We observed a predominance of MHC IIa and/or IIx in the vastus lateralis muscles of some subjects with SCI, which is consistent with previous studies demonstrating considerably lower proportions of type I fibres in SCI vastus lateralis muscles [1, 4] compared with non-paralysed muscles.

It is interesting to note, however, that three subjects expressed relatively high levels of MHC I (between 36% and 63%). In support of our observations, a recent study demonstrated extremely high expression (>99%) of MHC I in the tibialis anterior muscle of one individual with a paraplegic lesion [11]. It is well known that the phenotypic expression of muscle fibres is highly dependent upon their functional requirements. Given that reduced muscle activity usually leads to transitions of MHC expression from MHC I to MHC IIx [18], it is surprising that even after complete spinal lesion some paralysed muscles still express these high levels of MHC I.

It was recently shown that alterations in muscles fibre composition start rather soon after the lesion but reach a new steady state after approximately 70 months [4]. All three subjects with high MHC I expression sustained their injury >10 years prior to the study; thus, it seems unlikely that the duration of the lesion is responsible for the high proportion of MHC I. Furthermore, it has been suggested that females have greater proportions of MHC I than males [24]. Because two of the three subjects were males, our results were probably not related to gender. Another possible suggestion is that frequent muscle spasms may be an adequate stimulus for preserving high levels of MHC I. However, in our subjects only occasional muscle spasms occurred and subject no. 1 (having very high levels of MHC I) experienced almost no spasms at all. Possible effects of low levels of neuromuscular activity or hormonal influences which are also known to affect MHC composition [23] can not be excluded but clearly, even after very long periods of complete SCI, human paralysed muscles can still exhibit a heterogeneous muscle fibre type pattern.

Earlier work has suggested that there is a weak correlation between MHC content and muscle enzymatic activities in human muscle [25] suggesting a large heterogeneity of metabolic properties across constituent muscle fibres. The metabolic capacity of the muscle would not so much be determined by the MHC content but rather by the levels of muscle activity. Furthermore, it seems also reasonable to suggest that those fibres that are

the most frequently activated would most likely have the highest oxidative capacity. Given that the (lower limb) muscles of our subjects were basically inactive it may be expected that enzyme activities would be rather similar across fibre types in these paralysed muscles. Indeed, Fig. 2 demonstrates a broad range of SDH activity across different fibre types, when all fibres of all SCI subjects are considered.

An important observation of this study is that a clear relationship exists between MHC expression of the muscle samples and functional properties of the paralysed quadriceps muscle. For instance, those individuals who showed a high degree of fusion in low-frequency force signals also expressed high levels of MHC I. Our results indicate that nearly 80% of the degree of fusion could be explained based on the relative proportion of MHC I. These results support the findings of previous studies suggesting a close association between muscle composition and speed characteristics of the muscle [2, 10, 12]. In addition to contractile speed, approximately 65% of fatigue resistance could be explained by SDH activity. Assuming that a higher SDH activity indicates greater oxidative capacity this would allow for a better energy production, which seems important to sustain fatigue.

#### Effects of training

Twelve weeks of daily low-frequency (10 Hz) electrical stimulation did not significantly affect the fibre diameter of the paralysed muscles. Although changes in fibre diameter can only provide qualitative information about changes in muscle cross-sectional area these results would suggest no changes in fibre areas. Given the improved strength of these paralysed muscles reported earlier [9] this might seem surprising. On the other hand, the training duration may have been too short to induce significant fibre hypertrophy and, moreover, the nature of training stimulus (i.e. long duration of low-frequency activation, thus low mechanical forces) would not specifically benefit fibre hypertrophy. This suggests that the increased muscle strength following training most likely results from other changes in muscle properties, such as adaptations in fibre pennation angle, connective tissue content, or processes of excitation-contraction coupling.

The reduced FOA (increased degree of fusion), as a result of low-frequency training in our subjects [9], suggests a change in myosin expression towards a slower phenotype, similar to that previously demonstrated in small mammals after chronic electrical stimulation [19, 22]. However, such change in fibre type expression could not be observed in the present study, confirming previous results on human paralysed muscles [20, 21]. There is evidence indicating that human muscles adapt slower and to a lower extent to altered activity than muscles in small mammals [28], and that the magnitude of the fast-to-slow shift in MHC phenotype increases with training duration [6]. It is possible that longer and/or more intensive exercise regimens are needed to increase the expression of

MHC I in paralysed human muscles. In fact, one study that has actually shown an increased proportion of type I muscle fibres [14] accompanying contractile slowing [26] involved 20-Hz electrical stimulation for up to 8 h a day and for a period of 24 weeks on the paralysed tibialis anterior muscle. On the other hand, proportional increases in MHC IIa have been reported in the vastus lateralis muscles of individuals with SCI following 6 months of leg cycle training induced by electrical stimulation [1]. The subjects in that specific study trained only two or three times a week, thus the total exercise time of this training would be around 40 h, which is comparable with the stimulation duration in the present study. It may thus be speculated that not only the net exercise time but also the total training duration, including rest periods, should be long enough to allow muscle adaptations. It has been suggested that training-induced adaptations in calcium-related processes precede alterations in MHC expression and affect the time to peak and half-relaxation time of skeletal muscle [17, 19, 22]. We speculate, therefore, that calcium-related processes such as a reduced calcium re-uptake by the sarcoplasmic reticulum gave rise to the reduced FOA following training observed in our subjects [9].

The exercise-induced changes in metabolism seem consistent with the observed effects of the training on fatigue resistance [9]. The nearly 80% increase in SDH activity of the trained quadriceps muscle suggests that this type of stimulation training increases the level of oxidative metabolism, confirming results of previous studies on able-bodied [7, 29, 30] and paralysed human muscles [5, 14, 21].

## Conclusion

The results of the present study demonstrate that although paralysed quadriceps muscles of individuals with SCI normally undergo dramatic adaptations in contractile and metabolic characteristics, some muscles can still exhibit relatively normal muscle properties. Even after long-standing paralysis, slow contractile speed and high resistance to fatigue can be preserved and expression of contractile proteins and metabolic capacities seem to underlie these functional characteristics.

Furthermore, no evidence was found for changes in processes that may underlie the increased strength and reduced contractile speed. However, increased fatigue resistance of paralysed muscles induced by 12 weeks of daily stimulation training seems to arise, in part, from an improved oxidative capacity.

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