Regulation of myosin heavy chain expression in adult rat hindlimb muscles during short-term paralysis: comparison of denervation and tetrodotoxin-induced neural inactivation

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Abstract The extent to which myosin profiles within adult fast and slow muscles are altered by short-term paralysis remains equivocal. We used an array of specific antibodies to identify adult and developmental MHC isoforms within EDL and soleus muscle fibers, and show a marked multiple expression of MHCs with a general shift towards slower and more energy efficient MHC profiles after 2 weeks of denervation or TTX nerve conduction block. Paralysis also induced marked expression of an embryonic MHC within most EDL cell types, and a subtle, paralysis-sensitive, expression of α-cardiac MHC within specific EDL and soleus extrafusal fibers. Comparison of treatment groups also permitted assessment of the relative influence of neural activity versus trophic factors on these isoforms, and confirmed activity as a major, but not sole, regulator of MHC expression.

Key words: Myosin; Immunohistochemistry; Activity; Trophic mechanism; Embryonic MHC; α-Cardiac MHC

1. Introduction

Adult mammalian skeletal muscle fibers differ widely with respect to their morphological, biochemical and functional characteristics, and are known to display remarkable plasticity in response to alterations in neuromuscular usage [1]. A major portion of this diversity and capacity to adapt to changing functional demands appears to reside in the muscle cell's ability to transcribe different isoforms of the myosin heavy chain (MHC) component of the myosin molecule. Recent immunological techniques have led to the identification of at least four major MHC isoforms in adult mammalian skeletal muscle: a type I or β-cardiac MHC and the fast IIa, IIx and IIb MHC [2], each encoded by a separate gene from the MHC multigene family [3]. The different MHC isoforms, which display distinct ATPase activities, appear to relate to the maximal velocity of muscle fiber shortening, and the energetic cost of tension development of single muscle fibers [4,5].

Previous converging lines of evidence suggest that the relationship among MHC composition, ATP hydrolysis and the physiological events involved with muscle contraction is uncoupled after complete short-term paralysis. Under such conditions, skeletal muscles rapidly express muscle regulatory factors, as well as metabolic, and other specific myofibrillar proteins, characteristic of a 'slower' phenotype [6–9], and display pronounced contractile slowing [10–13]. Despite these marked and rapid transformations, it has generally been assumed that the myosin molecule is resistant, or slow to adapt, to complete disuse [10,14,15]. Furthermore, of those studies reporting changes, the direction of MHC isoform conversions within paralyzed fast or slow muscles remains equivocal and appears to depend upon the experimental method as well as type and developmental stage of the muscle at the onset of disuse [16–21]. Similarly, the extent to which the appearance of developmental configurations of MHC [14–17], or more recently, the α-cardiac MHC [22], are involved in extrafusal muscle fiber responses to paralysis has yet to be resolved.

In the present study, we used an array of 12 monoclonal antibodies to examine the expression of α, I, IIa, IIx, IIb, Emb and developmentally regulated type I MHC isoforms in EDL and soleus muscle fibers after a period of short-term paralysis induced by denervation or pharmacological blockade of sciatic nerve action potentials with tetrodotoxin. Comparison of these two models of complete disuse also provided insight into the relative contribution of nerve-evoked activity and putative chemical factors on the expression of these proteins, since during TTX paralysis, axon transport and the integrity of synaptic contacts are maintained [8,23,24]. Such an assessment appears timely since the extent to which trophic mechanisms influence myofibrillar proteins remains equivocal [14,25–29].

2. Materials and methods

2.1. Experimental design and methods of paralysis

Female Sprague-Dawley rats (200–230 g) were assigned to one of control ($n=7$), denervated (DEN; $n=9$) or tetrodotoxin-treated (TTX; $n=10$) groups. All surgical procedures were performed under aseptic conditions on animals anaesthetized with pentobarbital sodium (35 mg/kg i.p.). The musculature in one hindlimb of DEN animals was paralyzed by excising a 5 mm segment of the sciatic nerve immediately distal to the sciatic notch. In TTX animals, paralysis was induced in one hindlimb as described previously [8,24,30]. Briefly, a silastic cuff was secured around the sciatic nerve distal to the sciatic notch and connected via silastic tubing to a mini-osmotic pump (Alza Corp., CA) placed subcutaneously on the animals back. This system delivered TTX (350 μg/ml) to the nerve over 2 weeks at 0.5 μl/h. The efficacy of the nerve conduction block was verified twice daily using established ankle reflex criteria [8,24,30], and prior to muscle excision by stimulating the sciatic nerve at supramaximal voltage proximal to the cuff. The viability of nerve-muscle contacts was assessed using histological and functional criteria as described previously [8,24,30].

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Abbreviations: ATPase, adenosine triphosphatase; Ig, immunoglobulin; EDL, extensor digitorium longus; BSA, bovine serum albumin; PBS, phosphate-buffered saline; DAB, 3,3'-diaminobenzidine tetrahydrochloride

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Control animals were implanted with a sham delivery system consisting of cuff and tubing without osmotic pump. After 2 weeks, EDL and soleus muscles were excised, quick frozen in isopentane precooled in liquid nitrogen, and stored at −80°C.

2.2. Immunohistochemistry

Serial 10 μm tissue cross-sections were cut from each muscle midbelly and incubated for 1 h at room temperature in a blocking solution consisting of 5% goat serum in a carrier solution of 5% BSA in PBS. The sections were subsequently incubated at room temperature in the appropriate dilution of specific monoclonal antibodies raised against α (BA-G5), IIa (SC-71), IIb (BF-F3), IIx and IIb (212F), Emb (F1.652), Emb, neonatal, IIx and IIb (RT-D9), against all MHC except type IIx (BF-35), and developmentally regulated (A4.840, A4.951 and N2.261) and adult (BA-D5, BA-F8) epitopes of I MHC [2,31–33]. The sections were then rinsed with PBS (3×10 min) and incubated for 2 h at room temperature in peroxidase-labelled goat anti-mouse IgG, or IgM in the case of BF-F3 and A4.840 (Sigma, St. Louis, MO). After a second PBS rinse, the bound secondary antibodies were visualized using DAB (Pierce, Rockford, IL) as a chromogen. For α MHC (BA-G5), procedures were identical except that tissues were pre-fixed in 95% methanol (−20°C) and incubated with biotin-conjugated IgG2b (Zymed, San Francisco, CA) as a secondary and in peroxidase-conjugated streptavidin (Vector, Burlingame, CA) prior to DAB.

2.3. Fiber classification based on MHC composition

Fascicles were randomly selected, each from within six regions of the midbelly cross-section. All fibers (>200/section) within these fascicles were identified across serial sections using a computer-assisted image analysis system [24]. Fibers were classified as expressing type I, α, IIa, IIx, IIb, or Emb MHC, or as hybrid fibers coexpressing multiple MHC (Fig. 1). Muscle MHC percent distributions were calculated and means compared using an ANOVA.

3. Results

3.1. Paralysis-induced expression of slower adult MHC isoforms

Both DEN and TTX paralysis induced a general yet distinct shift in the expression of adult MHC towards slower isoforms within each muscle type. In the paralyzed EDL, the percentage of fibers expressing IIb MHC exclusively was ~40% lower than control (Figs. 2 and 3A), whereas the proportion

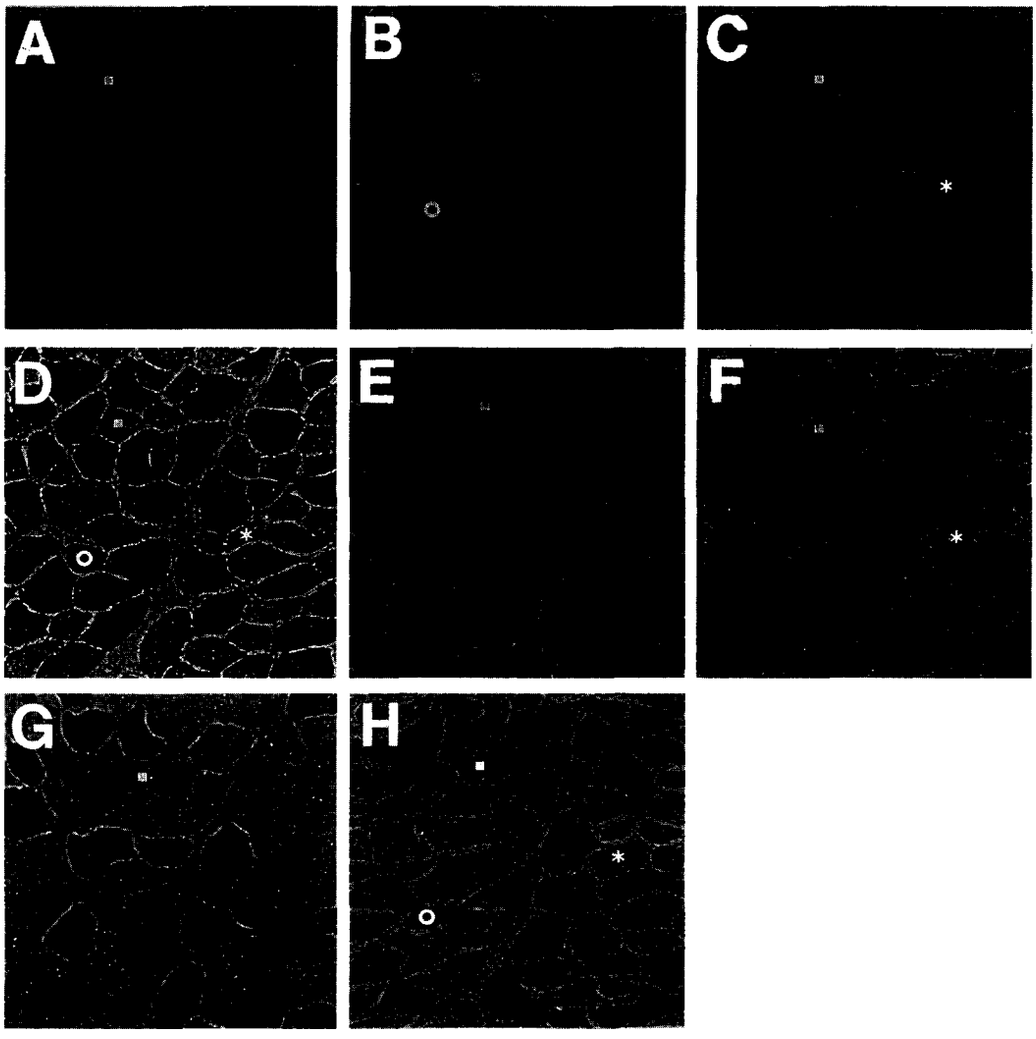

Fig. 1. Immunohistochemical detection of the various MHC isoforms in denervated EDL muscle fibers. Serial transverse sections were stained with monoclonal antibodies against MHC isoforms (A) I, (B) IIa, (C) Emb, (D) Emb, neonatal, IIx, IIb, (E) IIx and IIb, (F) all except IIx, (G) IIb and (H) α-cardiac. Note that in this figure all antibodies, including BA-G5 for α MHC, were visualized with indirect immunoperoxidase staining. Hybrid fibers coexpressing two or more different MHC isoforms are labelled: Emb/IIa (open circles), Emb/IIa/IIx (open triangles), IIx/IIb (open squares), Emb/I (closed squares), and Emb/IIx (asterisks) MHC are shown. Scale bar = 75 μm.

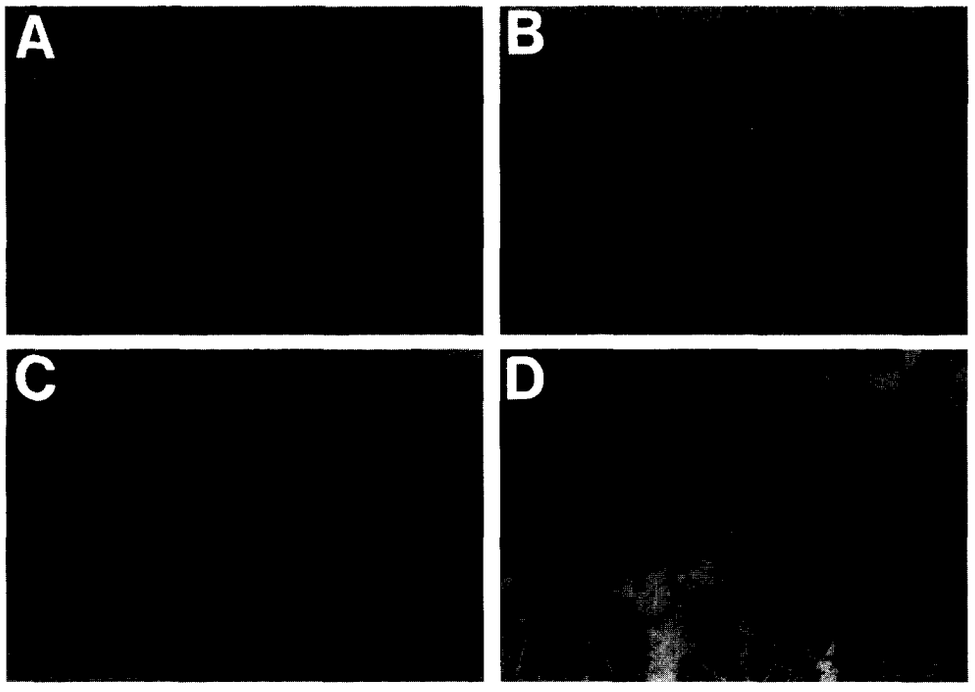

Fig. 2. Immunohistochemical detection of type IIb MHC in cross-sections of control (A) and denervated (C) EDL muscle fibers and detection of type I MHC in sections of control (B) and TTX (D) soleus muscle fibers. Note that fewer EDL fibers stained for IIb MHC in C compared to A, and that a larger number of soleus fibers stained for I MHC in D than in B. Scale bar = 300 μm for A,C. Scale bar = 200 μm for B,D.

of fibers expressing IIa MHC either alone or with other isoforms was 30% higher (Table 1, Fig. 3A). Compared to 10% in control, 40% of all paralyzed EDL fibers were hybrids coexpressing 2–4 different MHC (Figs. 1 and 3A). Although the proportion of EDL fibers exclusively expressing type I MHC was not different among groups, coexpression of this isoform with Emb or other fast MHC was observed after paralysis (Fig. 3A).

In paralyzed soleus muscles, there was an average 25% increase in the percentage of fibers expressing type I MHC (Table 1, Fig. 2), including hybrid fibers coexpressing this isoform (Fig. 3B). This appeared to be the result of a shift from a fast to slow phenotype within single fibers, since the proportion of cells expressing IIa MHC was 88% lower after paralysis (Table 1, Fig. 3B). Despite this trend, approx. 15% of paralyzed soleus fibers expressed IIx MHC, usually in conjunction with other isoforms (Fig. 3B). IIx MHC was rare in control soleus muscle fibers (1–2/section) and coexpressed with IIa.

Extrafusal fibers expressing type I MHC in control soleus, and I or IIb in control EDL, coexpressed faint but detectable amounts of α MHC, an isoform abundant in intrafusal fibers within these same muscles (Fig. 4). After short-term paralysis, immunolabeling of the α MHC within EDL fibers expressing I or IIb MHC was stronger, whereas in the soleus, expression of α MHC appeared to be coordinately up-regulated with that of I MHC.

3.2. *Expression of developmental MHC isoforms during paralysis*

Approx. 15% of control soleus fibers, mostly type IIa, expressed Emb MHC, whereas this isoform was absent in con-

Table 1
Expression of major MHC types in EDL and soleus muscles

		% fibers expressing MHC				
	n	I	IIa	IIx	IIb	Emb
Soleus						
CON	7	73.8 (2.4)	29.5 (1.8)	–	–	14.2 (5.6)
TTX	10	94.1 (2.0)[a]	3.9 (1.3)[a]	12.3 (1.3)[a]	–	5.5 (1.3)
DEN	9	90.6 (2.7)[a]	3.4 (2.8)[a]	17.5 (2.9)[a]	–	17.5 (2.9)[b]
EDL						
CON	7	3.0 (0.4)	21.5 (3.0)	39.8 (1.7)	51.0 (3.2)	–
TTX	10	5.7 (1.1)	26.0 (2.2)	45.0 (3.6)	40.8 (2.7)[a]	31.5 (4.0)[a]
DEN	8	4.2 (0.3)	30.2 (2.7)[a]	50.5 (3.7)	32.2 (3.6)[a]	27.7 (9.6)[a]

Values are means (S.E.M.).
[a]Denotes a significant difference from control ($p < 0.05$).
[b]Denotes a significant difference from TTX group ($p < 0.05$).

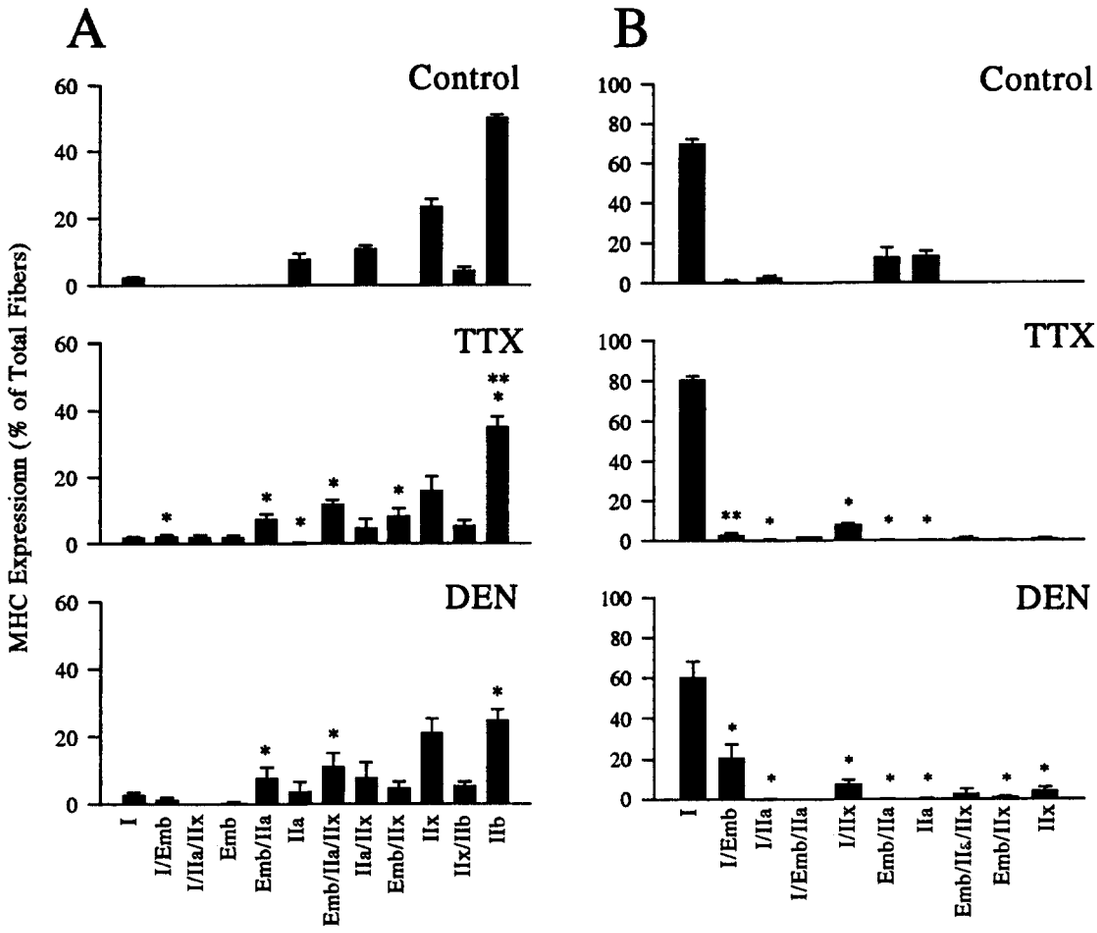

Fig. 3. MHC expression within EDL (A) and soleus (B) muscles after control, TTX and DEN conditions. Values are means ± S.E.M. MHC isoforms are arranged from left to right along the abscissa in the purported transition sequence of adult type MHCs [1,22]. Note that expression of the α MHC is not shown. *Denotes a difference from control ($p < 0.05$). **Denotes a difference from DEN ($p < 0.05$).

trol EDL (Table 1, Fig. 3A). On the other hand, ~30% of paralyzed EDL fibers expressed Emb MHC with I, IIa, or IIx, but rarely with IIb MHC (Table 1, Fig. 3A). The proportion of soleus fibers expressing Emb MHC after paralysis did not differ from control, and was expressed across all cell types. Regardless of treatment, EDL and soleus fibers displayed adult rather than developmental I MHC staining characteristics. Specifically, we did not observe any permutation of fibers that stained negative with at least one, but not all, of the three antibodies recognizing separate epitopes of I MHC sequentially expressed during development [31] (data not shown).

3.3. *Nerve-dependent regulation of MHC expression*

The contribution of electrical activity versus trophic factors was assessed by comparing MHC isoform distributions after denervation and TTX conditions. As with our previous findings [30], both treatments caused a similar loss in EDL (~40%) and soleus (~50%) muscle mass. Changes in MHC distribution profiles between experimental conditions were generally comparable (Fig. 3). Nevertheless, subtle differences in MHC expression between treatments were apparent and included a significantly lower proportion of fibers expressing exclusively IIb MHC in DEN than TTX-treated EDL (Fig. 3A), and a larger percentage of soleus fibers expressing Emb MHC after DEN (17%) compared to TTX (5%) counterparts (Table 1).

4. Discussion

In the present study, we report a rapid shift in the expression of prevailing adult MHC isoforms in both fast and slow muscles towards those displaying slower and presumably more efficient energy utilization characteristics after 2 weeks of short-term paralysis. We also show marked multiple expression of MHCs, as well as a subtle yet distinct paralysis-sensitive incorporation of the α-cardiac isoform, within specific sub-types of EDL and soleus extrafusal fibers. In the EDL, complete disuse also induced a rapid and significant expression of Emb MHC across most cell types.

Our results are the first to be consistent with reports of rapid, paralysis-induced, contractile slowing, and decreases in Ca^{2+}-activated myofibrillar ATPase activities in these muscle types [7,9–12], suggesting that the relationship among MHC isoforms, intrinsic rate of fiber shortening, and myofibrillar energy utilization is maintained during early post-paralysis. Our findings for the EDL, although consistent with others [18,20,21], are quite modest compared to the almost complete type I transformation of the rabbit gastrocnemius 2 months subsequent to early (8 days) postnatal denervation [16], suggesting that the MHC response to this condition may be species-, muscle- or developmental stage-specific. Similarly complex is the soleus response to paralysis, since the overall increase in I MHC was also accompanied by

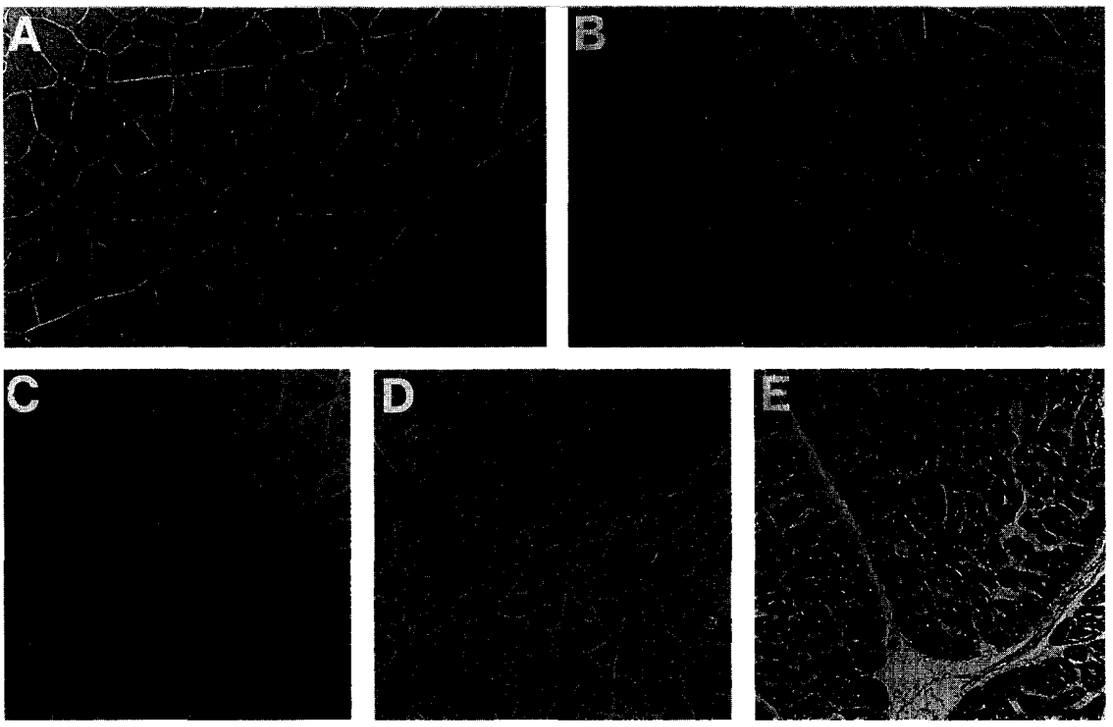

Fig. 4. Labeled avidin-biotin immunohistochemical detection of α MHC in the EDL after control (A) and DEN (B) conditions. We observed a subtle expression of this isoform in cells also expressing I (straight arrows) or IIb (b) MHC in control EDL, and a stronger labelling of α MHC within these cell types after DEN. Immunolabeling of control (C) and DEN (D) soleus indicated that expression of α MHC was associated with that of I but not IIa (a) MHC and was upregulated with I MHC during paralysis. Note that only two examples of cells expressing I, IIb or IIa MHC are shown in A–C. Also note the strong labelling of α MHC in adult rat intrafusal (curved arrows) and cardiac ventricular (E) muscle fibers. Specificity of the BA-G5 antibody for α MHC [33] was confirmed in the present study using Western blotting (data not shown). Scale bar = 75 μm.

an induction of the IIx MHC within a subpopulation of fibers. Indeed, a more substantive paralysis-induced replacement of slow MHC by fast counterparts may well be a long-term (>4 weeks) consequence of this condition [18,20,21], or when observed after short-term cordotomy [19], reflect the residual muscle activation inherent with this model [34].

Generally, MHC conversions are proposed to occur to accommodate cellular ATP requirements associated with altered contractile demands [1,35]. However, it is now apparent that MHC transformations can occur during paralysis, in the absence of overt fiber contractile and EMG activity [8] and when ATP requirements are very low [7,9]. In light of this and the finding that changes in fiber metabolic enzyme profiles appear to precede myosin isoform switching [35,36], it is possible that changes in enzyme levels influence the expression of specific contractile proteins. Indeed, the time course and direction of changes in fiber MHC expression appear to coincide with rapid decreases in the activities of enzymes of predominant metabolic pathways within individual paralyzed cells [8]. For instance, fibers which in control display high glycolytic and low oxidative profiles (i.e IIb) lose 50% of their glycolytic and maintain their oxidative enzyme activities during paralysis, thereby presenting metabolic profiles in the range of IIa-IIx fibers [8]. From the onset of paralysis, the progressive changes in metabolic enzymes may sequentially activate more than one MHC gene as specific putative metabolic thresholds are attained [35]. Such a process could explain the expression of multiple MHC in a significant number of paralyzed cells.

Muscle paralysis has been shown to induce the reexpression of developmental isoforms of the MHC [14,17], usually in IIa fibers and in amounts suggested to be too small (~1% of total MHC) to be functionally relevant [14]. Using a different antibody, we report a substantially greater expression of Emb MHC in the EDL in response to short-term paralysis, such that 30% of fibers expressed this isoform, suggesting that this may represent another distinct isoform of Emb MHC (cf. [37]). Although these results suggest that activity is a major repressor of Emb MHC in this muscle, it may not be the sole modulator since the mRNA for this isoform reappears in skeletal muscles after stretch [38], functional overload [39], or hypothyroidism [3]. Alternatively, since fibers displaying developmental MHC isoforms have lower ATPase activities and slower rates of tension development than those with adult fast MHC [40], their incorporation may be better suited to the changing metabolic profiles of specific cell types, and may be of functional relevance, during periods of muscle transformation [38].

In contrast to the EDL, expression of the Emb MHC in the soleus did not appear to be increased as a result of paralysis. Indeed, the expression of this protein persists within specific control soleus fibers until 16 weeks postnatal [39], suggesting that the concurrent activation of a developmental isoform replacement program and paralysis response elements may have rendered most soleus fibers refractory to its reexpression. It is also possible that an early transient elevation in Emb MHC went undetected in this muscle.

The α-cardiac MHC, a major isoform of rat heart and

intrafusal fibers, is known to be present in low amounts in specialized skeletal muscles such as the masseter and diaphragm [22]. Until now, evidence for the existence of this protein in hindlimb extrafusal fibers has been lacking. Using a proven specific antibody for this isoform [33], we were able to detect a subtle expression of α MHC within control hindlimb muscle fibers also expressing I or IIb MHC. We also provide evidence of a stronger incorporation of α MHC in response to paralysis within these cell types in the EDL and a coexpression with I MHC in transforming fibers in the paralyzed soleus. Our results coincide with a recent report [22], showing that α MHC transcripts are expressed at low levels in a variety of rabbit hindlimb muscles, particularly in those which express significant I MHC, and at higher levels in fast hindlimb muscles undergoing MHC transformations in response to chronic low frequency electrical stimulation [22]. As a result, the α MHC is proposed as an additional element in the sequential IIa to I fiber transitions that occur under these conditions [22]. Although the correlated appearance of α and I MHC in the short term of paralysis may support this contention, it is not clear from our results why the α MHC would be expressed with IIb MHC. Alternatively, the increased expression of α MHC during paralysis may occur in response to mechanical cues associated with the loss of contractile activity, as shown in cardiac myocytes [41].

Comparison of the expression of MHC after denervation and TTX paralysis permitted the assessment of the influence of trophic and activity-related mechanisms in the regulation of these proteins [14,25]. Consistent with previous studies [14,25,30], changes in muscle mass and distribution profiles of the various MHC isoforms were generally not different between experimental groups. However, we did observe subtle differences in MHC expression between treatments, suggesting a minor role for nerve-derived chemicals in the regulation of this protein. The lack of changes in the order observed in studies where the administration of trophic substances attenuated certain denervation-like changes upon myofibrillar proteins may be reconciled by the fact that in these reports the effect may have been related to the high systemic concentrations of these exogenous substances [28,29].

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