Changes in the Cholinergic System of Rat Sciatic Nerve and Skeletal Muscle Following Suspension Induced Disuse[1]

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ABSTRACT

Muscle disuse induced changes in the cholinergic system of sciatic nerve, slow twitch soleus (SOL) and fast twitch extensor digitorum longus (EDL) muscle were studied in rats. Rats with hindlimbs suspended for 2-3 weeks showed marked elevation in the activity of choline acetyltransferase (ChAT) in sciatic nerve (38%), in SOL (108%) and in EDL (67%). Acetylcholinesterase (AChE) activity in SOL increased by 163% without changing the molecular forms pattern of 4S, 10S, 12S, and 16S. No significant ($p > 0.05$) changes in activity and molecular forms pattern of AChE were seen in EDL or in AChE activity of sciatic nerve. Nicotinic receptor binding of ^{3}H-acetylcholine was increased in both muscles. When measured after 3 weeks of hindlimb suspension the normal distribution of type I fibers in SOL (87%) was reduced (to 58%) and a corresponding increase in type IIa and IIb fibers as seen. In EDL no significant change in fiber proportion was observed. Muscle activity, such as loadbearing, appears to have a greater controlling influence on the characteristics of the slow twitch SOL muscle than upon the fast twitch EDL muscle.

INTRODUCTION

Motor innervation plays an important role in the regulation of many properties of skeletal muscle. Elimination of the influence of the nerve by surgical denervation results in a variety of changes such as atrophy, a decrease in AChE and ChAT activity and an increase in chemosensitivity away from the endplate area in skeletal muscle (16).

The motor nerve is thought to supply influences that normally prevent these denervation changes. The question of how these influences of the nerve are mediated has been a matter of continuing controversy. Nerve transection has two effects: by preventing nerve impulses from reaching the neuromuscular junction it produces disuse of muscle, and by interrupting axonal transport it eliminates the release of trophic substances (17). These two components are not necessarily independent, since changes in impulse traffic may alter the rate or type of material that the axon releases.

Results of previous studies on the role of disuse have been inconsistent, partly due to the differences in muscles being used and the variety of procedures to reduce muscle activity. Disuse as previously produced by tenotomy (28), spinal cord section (7), joint fixation by pinning (12,27), casting (3) or bracing (10), general anesthesia (7) and prolonged blockade of nerve conduction by tetrodotoxin (5) reduce muscle activity, however, involve manipulations that may interfere with muscle tension or the integrity of the reflex arc and may involve traumatization of the hindlimbs and degeneration of motor neurons.

We are reporting on studies using a recently introduced technique of reduced loadbearing (24) which does not involve surgical manipulation in the region of muscle and nerve and allows a full range of joint movements. This

model involves suspension by the tail and allows locomotion by the forelimbs with rotational as well as longitudinal movements. Suspension can be maintained for four weeks without any sign of skin damage.

This model was used in order to determine to which extent the changes in denervated skeletal muscle are caused by disuse alone. The muscles chosen, the fast twitch extensor digitorum longus (EDL) and slow twitch soleus (SOL) differ not only in the number of impulses they receive during a 24 hour time period and in their contraction time (4), but also in AChE activity and pattern of the molecular forms of AChE (8,14,15).

METHODS

Animals

Male Sprague Dawley rats of matched age and weighing between 180-200 g, were used in this study. They were housed individually in separate cages, and in a temperature, humidity and light controlled room (22 $\pm$ 1°C; 50 $\pm$ 10% humidity; 12 h light/12 h dark cycle). To produce experimental muscle disuse, rats were suspended by their tails (9,25) such that their rear limbs were completely unloaded with the forelimbs carrying the load. In this model, animals were free to move and ate and drank ad libitum. During the observation period no signs of skin and tissue damage were seen. Non-suspended rats acted as controls and were housed in the same environment. To study the time course of biochemical changes, suspension lasted for periods varying from one to three weeks. At the specified intervals rats were sacrificed and sciatic nerve and soleus and EDL muscles were excised, weighed and prepared for analysis.

Biochemical Analysis

Choline acetyltransferase (ChAT, acetyl CoA choline O-acetyltransferase, E.C. 2.3.1.6.): The activity of ChAT was measured in sciatic nerve and in both soleus and EDL muscles employing the radiochemical assay of Fonnum (13). Sciatic nerves were pooled from both legs (1 cm length at the level of sciatic notch), minced on ice, and homogenized for 2 min with a Branson Cell Disruptor using microprobe (setting 6) in 19 volumes of 10 mM-EDTA, pH 7.4 having 0.5% Triton X-100. Soleus and EDL were freed from tendons, minced and homogenized for 1 min with Polytron (setting 6) in 19 volumes of EDTA-Triton X-100 (as described above). The enzyme activity was calculated as nmol ACh formed/total muscle protein/h or per mg protein in sciatic nerve/h.

Acetylcholinesterase (AChE, acetylcholine (acetyl-) hydrolase; E.C. 3.1.1.7.): The activity of AChE was determined according to the method of Ellman et al. (11). Homogenates were preincubated with tetramonoisopropylpyrophosphortetramide (iso-OMPA, 1×10^{-5} M) a specific inhibitor of cholinesterase (E.C. 3.1.1.8.) activity, for 30 min and then assayed with acetylthiocholine (3×10^{-3} M) as substrate. The different molecular forms of AChE were separated by velocity sedimentation following the modified methods of Massoulie et al. (22) as described by Groswald and Dettbarn (14) and were quantitated with the radiometric assay (18).

The enzyme activity of AChE has been calculated as nmoles acetylthiocholine hydrolyzed/total muscle protein/h, and also expressed as percent change compared to non-suspended controls.

^{3}H-Acetylcholine Binding: The isolated muscles were minced on ice and homogenized with polytron (setting 6) for 1 min, in 19 volumes of 50 mM-Tris HCl buffer (pH 7.4) containing 1 mM-$MgCl_2$, 120 mM-NaCl, 5 mM-KCl, 2 mM-$CaCl_2$, 0.1 mM phenylmethyl sulfonyl fluoride (PMSF), 0.1 µg/ml pepstatin, 0.1 µg/ml

aprotinin and 1.5 μM-atropine sulfate. The homogenate was passed through nylon monofilament mesh (105 μ opening) and centrifuged for 15 min (40,000 xg) and the supernatant was discarded. The process of homogenization and centrifugation was repeated in fresh buffer. To the final pellet, Tris HCl buffer containing 0.1% Triton X-100 to solubilize acetylcholine receptors and 0.1 mM diisopropyl fluorophosphoridate (DFP) to inhibit AChE, was added. The pellet was dissolved at two stages: first with polytron for 10 s (setting 6) and second time with Branson Cell Disruptor for 30 s (setting 7) and passed through nylon mesh (30 μ opening). The homogenate was allowed to stand for 30 min to assure complete AChE inhibition.

The binding assay technique was basically the same as described recently for brain (26), with some modifications as deemed necessary, due to muscle tissue. No attempts were made to separate endplate rich regions from endplate free regions. Briefly, the solubilized membrane preparation was assayed for nicotinic receptor binding using ^{3}H-ACh (sp. activity 80 Ci/mmol). The receptor assay mixture consisted of 40 nM-^{3}H-ACh, 50 mM-Tris HCl buffer and of 1 mM-carbachol. The binding reaction was started by adding 200 ul of 5% homogenate and incubated for 40 min at 0^{o}C. The reaction was terminated by vacuum filtration using millipore manifold filtration. Whatman GF/C glass fiber filters presoaked with 0.05% polyethylimine in Tris-HCl buffer were used for binding. The radioactivity was counted in 10 ml liquiscint using Beckman LS-250 at an efficiency of 60%. Each sample was assayed in quadruplicate, with and without carbachol. The specific binding was defined as the mean difference between the binding found with and without carbachol. The results were expressed as pmol/g tissue.

^{3}H-ACh was synthesized by esterification of methyl-^{3}H-choline chloride (80 Ci/mmol), as detailed earlier by Schwartz et al. (26). Protein was measured by the method of Lowry et al. (19) using bovine serum albumin as standard.

Histochemical Examination of Muscle Fiber Types: Animals were sacrificed and the SOL and EDL were removed from both sides and prepared for histochemical studies. Muscles were frozen in liquid nitrogen and 10 μ sections were cut on a freezing microtome. These were then reacted for actomyosin ATPase (20,21) which allowed identification of type I, IIa and IIb fibers.

Materials: The following chemicals were used: diisopropyl fluorophosphoridate (DFP), Aldrich Chemical Company, Inc., Milwaukee, WI; Triton X-100, Grove Village, ILL; Liquiscint, National Diagnostics, Somerville, NJ; atropine sulfate, carbachol, pepstatin, aprotinin and phenylmethyl sulfonyl fluoride, iso-OMPA, Sigma Chemical Co., St. Louis, MO; and radiochemicals: 1-^{14}C-AcCoA (45-50 mCi/mmol), ICN Chemical and Radioisotope Division, Irvine, CA; and methyl-^{3}H-acetylcholine iodide (50-100 mCi/mmol), New England Nuclear, Boston, MA; methyl-^{3}H-choline chloride (80 Ci/mmol), Amersham Corporation, Arlington Heights, ILL. During the course of this investigation, ICN Chemical and Radioisotope Division were unable to continue to supply 1-^{14}C-AcCoA, consequently this isotope was purchased from Amersham Corporation for some experiments. Nylon monofilament mesh was purchased from Small Parts, Inc., Miami, FL.

Statistics: Data were subjected to statistical analysis employing unpaired two-tailed Student's t test.

RESULTS

Biochemical Changes: Data presented in Table 1 indicates that ChAT activity in sciatic nerve showed a slight increase, though insignificant ($p > 0.05$) at the end of one week of suspension. Two weeks of suspension caused significant ($p < 0.05$) and maximum enhancement of this enzyme in sciatic nerve (38%). Thereafter, the enzyme activity gradually declined though remained significantly ($p < 0.05$) elevated compared to control. The ChAT activity in SOL and EDL muscles (intramuscular nerve fibers) was increased by 93% at the end of two weeks of suspension (Table 1), and remained at this level up to three weeks.

In non-suspended control animals, the AChE activity in EDL was found to be about twice to that of the SOL (Table 2). In suspended animals, the activity of EDL-AChE did not show a significant change when compared with non-suspended controls, however, AChE activity in soleus increased by 127% and 163% during second and third week of suspension, respectively. Moreover, when individual molecular forms of AChE in SOL were assayed, an increase of activity of all four major forms: 16S, 12S, 10S and 4S was found in SOL whereas, in the EDL, no significant change ($p > 0.05$) was observed in the 4S and 16S while the 10S was increased (Fig. 1). The contribution of the individual forms to the total activity was not different from that of control (Table 3). AChE activity in sciatic nerve was not affected significantly up to three weeks of suspension compared to non-suspended controls (data not shown).

Data presented in Table 4 on nicotinic acetylcholine receptor binding using ^{3}H-ACh as ligand indicate that two weeks suspension caused a twofold increase ($p < 0.05$) in ligand binding in solubilized membrane preparations prepared from both EDL and SOL compared to control. No attempts were made to separate tissue in end plate and non end plate regions.

The Changes Related to Fiber Types: The cross sectional area of SOL and EDL were significantly reduced when sections were made at mid length through the muscle belly. The type I fibers of the SOL were most affected by muscle disuse, while the same type of fibers in the EDL were less reduced in size.

The fiber type distribution showed also changes with disuse. In SOL, the proportion of fibers staining as type I fibers was reduced from 87% in control to 58%, while IIa fibers proportion increased from 2% to 18% and IIb fibers from 11% to 25% of total fibers population. The EDL, a muscle that has very few type I fibers to begin with, exhibited no significant change in fiber type proportion.

DISCUSSION

The present experiments were designed to determine the extent of disuse induced changes in AChE, ChAT and AChR in slow and fast muscle. We found (1) that the changes induced by disuse in regard to ChAT and AChE do not resemble those seen with denervation, (2) the changes seen in AChR numbers are in qualitative agreement with those seen following denervation, and (3) that the slow SOL is more responsive to disuse than the fast EDL muscle. These data provide additional evidence that the role of use and disuse of a given muscle in the regulation of AChE may be different in slow muscle and fast muscle.

Regulation of ChAT in nerve and muscle. The data presented in Table 1 demonstrate that ChAT was significantly enhanced in sciatic nerve and in both skeletal muscles (SOL and EDL) during two to three weeks of hindlimb suspension. Whether this increase is solely due to the intramuscular nerve fibers and/or to the muscle fiber proper remains to be seen. The increase of ChAT activity observed in sciatic nerve during suspension appears to be related to an increase in axoplasmic transport of this enzyme, since in preliminary data a 30% increase in the rate of transported ChAT was found (Gupta-Dettbarn). Whether its synthesis is increased as well remains to be seen. The observed increase in ChAT could be in response to the increase in AChR seen in disused muscle (see below). The increase in ChAT activity does not agree with the findings of earlier studies of muscle disuse. Pinning of the hindlimb for seven days (27) caused a decrease in ChAT in disused gastrocnemius muscle, while Butler et al. (5) observed no change in ChAT activity in the sciatic nerve, anterior roots or intramuscular terminals from SOL and EDL when blockade of nerve conduction was induced by repeated subperineural injections of tetrodotoxin (TTX). Following surgical section (5) or crush of sciatic nerve (8), a decrease of ChAT activity was found in proximal and distal nerve and muscles. The difference may be due to the models of disuse and duration of disuse.

Regulation of AChE in nerve and muscle. The significant increase in AChE activity in SOL as compared to the EDL may in part be related to changes in the white/red muscle fiber ratios within the SOL since white fibers have higher enzyme activity (see below). This seems unlikely, however, since the pattern of molecular forms resembles that of the control soleus and no adaptation of EDL patterns was observed. In addition, differences may be

found in the muscles themselves which may control their characteristics by substances whose availability may depend on the level of contractile and metabolic activity.

The lack of effect of disuse on AChE activity in EDL and the increase of this enzyme in SOL is in marked contrast to the findings of Butler et al. (5). These authors found a loss of AChE activity in SOL and EDL with TTX induced disuse similar to that occurring after denervation. Disuse induced in hemidiaphragm with spinal hemisection at C2 (17) did not cause significant changes in endplate or non endplate AChE in this particular muscle.

Some of the differences may be found in the types of muscles used, such as gastrocnemius, diaphragm, SOL, and EDL which may respond in different ways to disuse. In addition, the technique of hindlimb suspension used in our study has certain improvements over the other techniques used previously. As outlined by Corley et al. (6), there is no muscle degeneration as seen after tenotomy, and the peripheral and central nervous system remains intact. It differs from limb immobilization produced by pinning, casting or bracing, since muscle contractions are isotonic, rather than isometric. A full range of joint movements is possible and stretching of muscles by gravitational forces is eliminated in our model.

Disuse and the AChR. While disuse affected mainly AChE of SOL with little or no change in the EDL enzyme, its effect on ACh binding was similar in SOL and EDL. The total number of receptors as measured by ACh binding increases by about 116-128%. This increase in receptors in innervated muscle may be related to the increase in ChAT activity found in nerves. Whether this increase reflects changes in non endplate regions only (1,2,23) remains to be seen. Experiments are in progress to study these changes in endplate and non endplate regions separately.

Disuse and fiber type. Suspension disuse caused changes in SOL actomyosin ATPase characteristics suggesting a conversion of 29% of the type I fibers to IIa and IIb fibers. In EDL, no significant changes in fiber type proportion were seen. Across all fiber types there was more disuse atrophy in the postural SOL than in the EDL. Type I fibers were more affected in the SOL and type II fibers were relatively more atrophied in the EDL.

It is obvious from these data that disuse affects the type I fibers differently from the type II fibers and the antigravity muscle such as the SOL shows a greater susceptibility to disuse and absence of weight bearing than the fast twitch muscle such as the EDL. This is confirmed by studies of disuse on the hindlimb of hamsters (6).

The muscles in our experiments were no load bearing but free to contract (isotonic contraction), thus there is a need for a load bearing to prevent atrophy. The lesser degree of atrophy in the EDL indicates that this muscle is normally not used as an antigravity muscle during locomotion, while the SOL is normally an extensor and antigravity muscle.

These data indicate that AChE and some cytochemical properties of skeletal muscle are strongly dependent on level of load bearing and patterns of motor unit activity. With disuse the usually slow SOL appeared to change its fiber type composition to one that more resembled a fast muscle. The AChE activity also increased, however the hypokinetic SOL retained its characteristic distribution of molecular forms of AChE. Since the EDL is already a fast muscle, disuse produced little change in AChE activity.

The reasons for the SOL showing this more pronounced sensitivity to inactivity are not clear, however its greater dependency on nerve regulated influences has been observed previously. These data suggest that in the absence of normal patterns of weight bearing activity and without trauma to

the lower motor neuron, fast and slow muscles may revert to a faster type. The characteristics of slow SOL muscle appears to be more dependent on activity related mechanisms and load bearing than the fast EDL. Further studies involving axoplasmic transport, release of ACh from nerve terminals, changes in contractile characteristics and careful monitoring of electrical activity during disuse will help to more clearly elucidate the role of the nerve and contractile activity in the maintenance of muscle.

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FIGURE LEGEND

Fig. 1A & B. Velocity sedimentation gradient separation of the acetylcholinesterase (AChE) forms from extensor digitorum longus (EDL) and soleus (SOL) muscles. Gradients contained two hundred micrograms of a high speed supernatant from the muscles (see Methods) which was layered on a 5-20% sucrose gradient and centrifuged at 35,000 rpm for 18 hrs. Approximately 55 fractions were collected and 0.03 ml was incubated for four hrs in the presence of (^{3}H)acetylcholine and iso-OMPA (10 μM).

A. Effects of three weeks of disuse on the SOL Δ-----Δ control muscle, o-----o hypokinetic muscle.

B. Effects of three weeks of disuse on the EDL Δ-----Δ control muscle, o-----o hypokinetic muscle.

Arrows indicate position of markers, G = β galactosidase (16.0S), C = catalase (11.1S), and P = alkaline phosphatase (6.1S).

TABLE 1

Effect of hindlimb suspension induced muscle disuse on choline acetyltransferase activity in rat sciatic nerve (nmole acetylcholine formed/mg protein/h) and slow soleus (SOL) and fast extensor digitorum longus (EDL) muscle (nmole acetylcholine formed/whole muscle protein/h).

		Duration of Suspension		
Tissue	Animal	1 Wk	2 Wk	3 Wk
Sciatic Nerve	Control	99.5±7.1	133.2±7.7	106.6±1.9
	Experimental	121.3±8.4 (+22)	184.1±4.2[a] (+38)	140.0±3.1[a] (+31)
Soleus	Control	4.1±0.2	4.2±0.4	3.9±0.1
	Experimental	5.5±0.2 (+34)	8.1±0.4[a] (+93)	8.1±0.4[a] (+108)
EDL	Control	3.4±0.2	3.8±0.1	3.6±0.4
	Experimental	4.4±0.2 (+29)	5.5±0.3[a] (+45)	6.0±0.2[a] (+67)

Each value is the mean ± SEM of choline acetyltransferase activity, obtained from 5-9 animals and number in parenthesis represents percent change compared to non-suspended control.

[a]indicates a statistically significant difference ($p < 0.05$) compared to control value at each corresponding time interval.

TABLE 2

Effect of hindlimb suspension induced muscle disuse on acetylcholinesterase activity (nmole acetylcholine hydrolyzed/whole muscle protein/h) in rat slow soleus and fast extensor digitorum longus (EDL) muscle.

		Duration of Suspension		
Muscle	Animal	1 Wk	2 Wk	3 Wk
Soleus	Control	285.9±33.4	266.4±14.13	275.7±23.0
	Experimental	358.3±45.1 (+25)	605.0±26.3[a] (+127)	726.1±58.7[a] (+163)
EDL	Control	510.9±43.4	466.2±30.6	467.0±39.0
	Experimental	450.1±49.3 (−12)	504.5±28.3 (+8)	393.7±24.3 (−16)

Each value is the mean ± SEM of acetylcholinesterase activity, obtained from 6-15 animals and number in parenthesis represents percent change compared to non-suspended controls.

[a]indicates a statistically significant difference ($p < 0.05$) compared to control value at each corresponding time interval.

TABLE 3

Activities of AChE molecular forms after three weeks of muscle disuse.

	SOL		EDL	
	Control	Disuse	Control	Disuse
4S	30±2	33±2	51±3	41±9
10S	19±2	20±3	27±3	36±6
12S	23±1	25±2	---	---
16S	28±3	22±3	22±1	24±3

Data are from four separate experiments and activities are expressed for each form as percentage of total recovered activity.

TABLE 4

Effect of hindlimb suspension induced muscle disuse (two week) on nicotinic receptor binding in soleus and extensor digitorum muscle.

Animal	Soleus	EDL
Control	1.29±0.16	1.92±0.19
Experimental	2.94±0.19[a] (+128)	4.15±0.33[a] (+116)

Binding of 40nM ^{3}H-acetylcholine was measured in the presence of 0.1 mM DFP, 1.5 μM atropine, 0.1 mM PMSF, 0.1 μg/ml pepstatin and 0.1 μg/ml aprotinin. Specific binding determined as total-nonspecific (in the presence of 1 mM carbachol) binding.

Each value is the mean ± SEM of three experiments and expressed as pmol/g tissue and number in parenthesis represents percent change compared to non-suspended control.

[a]indicates a statistically significant difference ($p < 0.05$) compared to control.