A STUDY OF PROTEIN METABOLISM IN DENERVATION AND REINNERVATION FOLLOWING SCIATIC NERVE CRUSH

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1. Introduction

The loss of proteins in denervation atrophy of skeletal muscles has been shown to result from increased degradation and decreased synthesis of proteins [1]. Upon reinnervation, atrophied muscles begin to restore their structure and function. Although physiological and morphological studies of reinnervated muscles have been carried out by many investigators, there is little information of how protein metabolism changes in response to reinnervation. It has been reported [2–5] that identical denervation effects on muscles are caused either by a single crushing or complete section of the sciatic nerve. In the cases of nerve crushing 15 days after operation, the signs of reinnervation were observed, and at the same time the increased autocatalytic degradation of proteins in the muscles began to decrease toward the normal level [5]. The present study was carried out to observe the time course of changes in protein metabolism in soleus and extensor digitorum longus muscle (EDL) after a single crush of the sciatic nerve.

During the first 2 weeks after nerve crushing, in vivo pulse-labelling (20 min) of proteins with [^{3}H]-leucine was reduced in myofibrils of both EDL and soleus. A slight reduction in soleus sarcoplasm, but not in EDL was observed. About 3 weeks after the operation, the protein labelling was increased (by 50–150%) in all muscle fractions, but returned to normal by the 5th week. At all times, the labelling of tRNA with [^{3}H]leucine remained constant.

Histological measurements in soleus and EDL were made of muscle cross-sectional area and the myofibrillar proportion of this area. The mean fiber area in both soleus and EDL showed a marked drop of approximately 40% of normal in the first 4 days, returning to normal levels by the 16th day. A fraction of myofibrillar areas to fiber area showed a little change throughout the period of observation. For the purpose of following the changes in muscle mass, techniques of both histology and autoradiography were employed.

2. Materials and methods

Male Wistar rats, weighing about 200 g on arrival, were maintained in our animal case facility supplied with food and water ad libitum for 1 week before the experiments. On the date of operation, the sciatic nerve of one leg was exposed under pentobarbital anesthesia with aseptic precautions. The exposed sciatic nerve was firmly crushed at the point of division into lateral peroneal and posterior tibial nerve. The skin sutured with fine silk was clipped to prevent the rats causing dehiscence of the wound by biting the suture.

Between 4 and 38 days after the operation, L-[4,5-^{3}H]leucine (100 μCi/100 g body weight; S. A. 0.4 Ci/mmol, Amersham Corp.) was injected intraperitoneally, and the rats were sacrificed by decapitation within 20 min after injection. The muscles – soleus, extensor digitorum longus (EDL), and in some cases, gastrocnemius – were dissected, weighed and placed in a chilled solution consisting of 0.25 M sucrose, 0.1 M Tris–HCl, 0.5 mM EDTA and 10 mM L-leucine, pH 7.2. Each muscle was minced with scissors and homogenized in a Polytron homogenizer by two 15 s bursts at the rheostat setting 5. The volume of homogenate was adjusted to give a 5% (muscle wet weight/ml of the homogenate) suspension. The homogenate was centrifuged in a Sorval HB-4 rotor at 1500 g for 15 min. The resulting pellet was washed 3 times with 0.1 M Tris–HCl (pH 8.0) containing 1 mM EDTA and 10 mM leucine

to obtain a myofibrillar fraction. The supernatant was further centrifuged in a Spinco rotor No. 65 at 100 000 × *g* for 40 min to prepare a soluble sarcoplasmic fraction. The protein concentration of each fraction was adjusted to 2–3 mg/ml. The proteins contained in each fraction were precipitated by 20% trichloroacetic acid (TCA) containing 10 mM leucine, collected by centrifugation and washed repeatedly with 10% TCA until the washing TCA became free of extractable radioactivity. Usually, 3 washes satisfied this requirement. The precipitate was dissolved first in a small volume of 1 N NaOH and diluted by an equal volume of H_2O. The protein content of the solution was measured by the Biuret method, and the radioactivity was counted using Aquasol (New England Nuclear Co.), the volume of which was 10 ml/0.2 ml of the dissolved proteins, in a LS200 Beckman Scintillation counter.

For the assay of leucyl-tRNA, the tissue was homogenized in a phenolsodium acetate (pH 4.0) mixture as described by Allen et al. [6]. Since the soleus and the EDL muscle were of small sizes (100 mg or less), the ethanol precipitation procedure of RNA was omitted. The extracted RNA was purified through twice repeated chromatography using Sephacryl 200 (Pharmacia Fine Chem. Co.). The condition of chromatography was as described by Martin et al. [7] who used Sephadex G-100 for this purpose. The tRNA eluted from the second chromatography gave the ratio of A_{260} nm/A_{280} nm of 1.7–1.8. Separated tRNA was analysed for its radioactivity in the same procedure as described for the protein associated ^{3}H-activity. The relative specific activity of tRNA was expressed in terms of counts per minute per one absorbance (at 260 nm) unit.

Small portions (1 mm × 5 mm) of muscle were obtained as soon as muscles were removed from the rats. After fixation in glutaraldehyde each specimen was diced and embedded in Epon after appropriate orientation. True cross-sections were obtained. These sections were studied by electron microscopy in a Siemens' L-102 electron microscope and the measurement of mean fibrillar fraction of fiber area and mean fibrillar area per fiber established as described by Stonnington and Engel [8].

3. Results and discussion

Although the denervation effects on muscle protein metabolism have been well documented [1,8–13], there is little information of how muscles regain proteins after reinnervation. To follow the changes in protein metabolism in muscles after the nerve-crushing operation in one leg, we measured the in vivo pulse ^{3}H-labelling of myofibrillar and cytoplasmic proteins of soleus and EDL dissected from the rats sacrificed at intervals after operation. The labelling data were first expressed as the relative specific activity (cpm/mg protein) of each protein fraction from the individual leg of a rat. Then, the ratio of the relative specific activities (the operated leg/the contralateral leg) was calculated and plotted against time in days after the nerve crushing (fig.1). The radioactive labelling data obtained from the sham operated animals indicated that the above ratio remains in a range from 0.95 to 1.08, at various times after operation. During the first 2 weeks after the nerve crushing, the labelling of proteins in soleus muscles of the operated legs was lower than the control, whereas in EDL, a slight decrease in myofibrillar

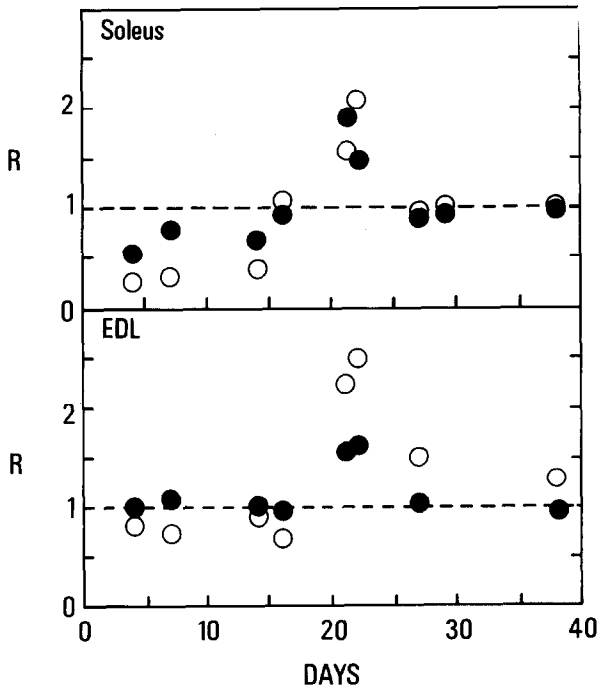

Fig.1. Time dependent changes of leucine incorporation into sarcoplasmic (•) and myofibrillar (○) proteins after the sciatic nerve crush. R represents the ratio of two relative specific activities; denervated leg muscle/contralateral leg muscle. The dotted horizontal lines indicate that the rate of radioactive labelling of proteins in the denervated leg muscle is equal to that in the control leg.

Table 1
Relative specific activity (cpm/absorbance) of tRNA labelled with [^{3}H]leucine

Days after denervation	Muscle	Denervated	Contralateral
4	Soleus	4950 ± 450 (2)[a]	4880 ± 450
	EDL	3340 ± 300 (3)	3300 ± 300
	Gastrocnemius[b]	3830 ± 350 (6)	3560 ± 350
22	Soleus	4560 ± 450 (3)	4420 ± 450
	Gastrocnemius	4100 ± 400 (6)	4050 ± 400
38	Gastrocnemius	3960 ± 400 (6)	4010 ± 400

[a] The numbers in parentheses indicate the numbers of samples tested
[b] Although gastrocnemius muscle was not used to measure the [^{3}H]leucine incorporation into proteins, its size allowed to experiment repeatedly for the assay of tRNA labelling, and the data were included to illustrate that tRNA labelling was constant during the period of observation

proteins was detected. About three weeks after the operation, the ^{3}H-labelling of proteins of both fractions was seen to be increased (100–150% above control) in solei as well as in the EDLs of the operated legs. In the beginning of the 5th week postoperation, the ^{3}H incorporation levels returned to control level.

As shown by Martin et al. [7] in their study of myosin heavy chain turnover in rat hearts, assessment of protein turnover rate by radioactive labelling techniques should be based on the measurement of radioactive precursor, i.e., aminoacyl-tRNA. If the leucyl-tRNA turnover in muscles is affected by denervation or nerve crushing, the ^{3}H incorporation into proteins may change accordingly. We, therefore, attempted to determine the [^{3}H]leucyl-tRNA level in the muscles used for the protein labelling determination. Table 1 shows that the leucyl-tRNA levels in muscles are not influenced by crushing sciatic nerve.

During the period of protein labelling (20 min), the [^{3}H]leucyl-tRNA level has decayed, but its specific activity is estimated to be still 50–60 times higher than the [^{3}H]leucine specific activity incorporated into proteins [7] and the [^{3}H]leucine incorporation into proteins is close to linear with time. If one assumes that average half-life of proteins is longer than 200 min, the observed incorporation may be considered as representing the average turnover rate. Since such an assumption cannot be made in this study, the results simply exhibit some changes in overall protein metabolism. Nevertheless, the increased [^{3}H]leucine incorporation into proteins occurring 3 weeks after nerve crushing appears to be a phenomenon hitherto unreported. Though its physiological meaning is not evident, this increased [^{3}H]-leucine incorporation seems to take place shortly after the signs of reinnervation and the decline of the autocatalytic protein degradation that is associated with nerve crushing [5]. In an earlier study, Gutmann and Young [15] observed that the reflex activity returns about 11 days after nerve crushing.

As is well known, denervated muscles lose myofibrillar proteins faster than sarcoplasmic proteins. In this study, histological measurements in soleus and extensor digitorum longus were made of the muscle fiber cross-sectional area and the myofibrillar proportion of this area. The mean fiber areas in both soleus and extensor digitorum longus show a marked drop to approximately 40% of normal during the first 4 days. The mean fiber areas rebound to normal levels by the 16th day. The fraction of myofibrillar areas show little change throughout the period of observation to the 20th day in either muscle. This latter observation may reflect differences in muscle fiber response to crushing of its nerve supply rather than nerve section as described by Stonnington and Engel [8]. The difference in regeneration time is also likely to be related to the transient nature of crushing nerves as opposed to permanent denervation and it seems that the major regenerative effort as judged histologically takes place between the fourth and the 16th day.

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