J. Anat. (1988), **158**, pp. 109–114
With 1 figure
Printed in Great Britain

The importance of stretch and contractile activity in the prevention of connective tissue accumulation in muscle

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(*Accepted* 18 *August* 1987)

INTRODUCTION

The loss of sarcomeres which results from immobilisation in the shortened position is accompanied by reduced muscle compliance (Tabary *et al.* 1972; Williams & Goldspink, 1978). It has been shown that there is an increase in the proportion of collagen in the immobilised muscles which could explain the increase in stiffness (Williams & Goldspink, 1984). An increase in connective tissue occurs after only a few days of immobilisation, i.e. before there is a significant loss of serial sarcomeres, implying that immobilisation has a direct effect on the connective tissue rather than the increase being due to redistribution of connective tissue following sarcomere loss. However, immobilisation in itself does not appear to be the main cause of the connective tissue changes since it is only when muscles are immobilised in the shortened position that the increase occurs (Williams & Goldspink, 1984). Thus the position in which the muscle is held seems to be an important factor in connective tissue accumulation and muscle stiffness.

When immobilised in a shortened position, a muscle is subjected not only to lack of stretch but also to reduced contractile activity. In order to determine which parameter is important in determining connective tissue remodelling, it was decided to investigate the effect of electrical stimulation on immobilised rabbit soleus muscle. In particular it was hoped to determine whether contractile activity prevents connective tissue accumulation in muscle immobilised in the shortened position.

Reduction of fibre length occurs not only when a muscle is held in a shortened position by immobilisation but also when it is working over a reduced range. This has been found in the diaphragm muscle of emphysematous hamsters where chronic pulmonary over-distension keeps the muscle fibres in a shortened position (Supinski & Kelsen, 1982). A loss of sarcomeres enables the muscle to generate more tension at shorter fibre lengths compared with the control, i.e. the fibres adapt to the functional length of the whole muscle in the same way as fibres from limb muscles adapt to immobilisation in a shortened position. It is not known, however, whether, in muscle which is working at a reduced functional length, there are also changes in connective tissue similar to those found in immobilised muscle. It was decided, therefore, to measure the connective tissue content of diaphragm muscles from emphysematous hamsters.

MATERIALS AND METHODS

Skeletal muscle/electrical stimulation model

Male rabbits of the strain NZW weighing approximately 2·5 kg were anaesthetised with sodium pentobarbitone administered intravenously. Teflon-coated stainless steel wire was used to form electrodes which were sewn into the muscle on either side of the sciatic nerve in the thigh. The wires were externalised on the back of the rabbit where they were connected to a miniature stimulator delivering impulses at a frequency of 5 Hz. Stimulation was carried out continuously for 7 days. The stimulation strength, approximately 0·5V, was adjusted so that it elicited muscle contraction but did not cause any noticeable discomfort to the rabbits.

The animals were divided into five groups, each of eight animals, the first serving as the control group. In Group II the soleus muscle was immobilised in a shortened position by means of a plaster cast holding the foot in plantar-flexion. In Group III immobilisation in the shortened position was combined with stimulation. In Group IV the muscle was immobilised in the stretched position and in Group V the muscle was stimulated only. After a 7 day period of stimulation, immobilisation or immobilisation combined with stimulation the animals were killed and the soleus muscles removed.

Three animals from each group were used for fibre length and sarcomere number determination. Muscles were fixed in glutaraldehyde, placed in 30% nitric acid for two days and then stored in 50% glycerol. A small bundle of the fibres inserting furthest onto the distal tendon was removed from each muscle and single fibres were dissected free and mounted in glycerine jelly. Five muscles from each group were used for connective tissue analysis. These muscles were excised and slices taken from the mid-belly region. The muscle portions were frozen in isopentane pre-cooled in liquid nitrogen. Cross sections 10 μm thick were cut and stained for connective tissue using picro-sirius red (Sweat, Putchler, Sanford & Rosenthal, 1964).

Diaphragm/emphysema model

Twenty four outbred male golden hamsters were divided into control and experimental groups. Animals from the experimental group were anaesthetised by CO_2 inhalation and treated with a single endotracheal instillation of porcine pancreatic elastase (200 mg/0·5 ml/100 gbw) (Hayes, Korthy & Snider, 1975). Six weeks or six months after treatment the animals were anaesthetised with sodium pentobarbitone and exsanguinated. The lungs of the six week animals were inflation-fixed by injecting 5 ml of 4% formalin–1% glutaraldehyde. The volume displacements of these lungs were determined by the method of Scherle (1970). Bundles of intact costal diaphragmatic fibres were dissected from the region surrounding the sites of insertion of right and left phrenic nerves. The origins from the ribs and the insertions on the central tendon were left intact. Fibre bundles from one side were pinned on cork, fixed in glutaraldehyde and then stored in 50% glycerol. Fibre bundles from the second side were supported on a piece of liver, frozen in isopentane pre-cooled in liquid nitrogen and sectioned on a cryostat at 10 μm before being stained for connective tissue.

Sarcomere number measurements

Single muscle fibres from the hamster diaphragm and from the rabbit soleus muscle were teased out and mounted in glycerine jelly. Using a projection microscope fibre lengths were measured. Average sarcomere length was determined by counting the number of sarcomeres/100 μm at 3 different points along the fibre. In this way total

sarcomere number could be estimated. Measurements were made on 3 muscles from each group and on 10 fibres for each muscle.

Connective tissue analysis

Picro-sirius red stains the connective tissue of the epimysium, perimysium and endomysium a bright red which, under a green optical filter, appears black and contrasts well with the pale muscle fibres. Sections from the hamster diaphragm muscle fibre bundles and the rabbit soleus muscles were examined with a Leitz microscope to which was attached a high resolution video camera. The video information was fed into a digitising interface (Computec diplomat) connected to a peripheral slot of a microcomputer (Apple II) which converted the video image into a computer graphic image with two selectable grey levels. Using a control slide the video image was adjusted by means of a threshold facility so that there was a good correspondence between the optical and video images. No further adjustments were made whilst taking readings of slides from all experimental and control muscles for a given group. Sections were scanned using a small area (approximately 0·02 mm^2) and readings taken of the connective tissue content. Five experimental and five control muscles were scanned from each group and approximately 10 readings were taken from each muscle. Results, expressed as the percentage muscle area stained, were compared using a one-way analysis of variance.

RESULTS

Rabbit soleus muscles

Muscles which were immobilised in a shortened position showed a loss of serial sarcomeres and an increase in the amount of connective tissue per unit area of muscle (Table 1; Fig. 1). In contrast, when immobilisation in the shortened position was combined with stimulation there was an even greater loss of serial sarcomeres but no change in the connective tissue content. Stimulation alone also caused a reduction in serial sarcomeres without concomitant connective tissue changes. When muscles were immobilised in the extended position there was an increase in serial sarcomeres; connective tissue concentration was unchanged.

Hamster diaphragm muscles

In experimental emphysema it has been shown that there is an increase in lung volume (Lucy, Snider & Javaheri, 1982). In the present study the mean lung volume displacement of the treated animals was 59% greater than in the control animals at 6 weeks post-treatment ($5{\cdot}0 \pm 0{\cdot}25$ ml in the experimental group compared with $3{\cdot}70 \pm 0{\cdot}19$ ml in the control group) thus confirming that emphysema had been induced in the experimental group. The number of serial sarcomeres along the length of the diaphragmatic muscle fibres from the two emphysematous groups was significantly less than in the control groups (Table 2). It can be assumed that the shortening of the diaphragm was not related to reduced body weight since it has been shown previously that, although emphysematous animals are significantly lighter than controls, nose-to-tail length and limb muscle length are unaffected (Supinski & Kelsen, 1982). In the present study the amount of fibre shortening was found to be greater in the 6 months group than in the 6 weeks group.

Connective tissue analysis showed a considerable age-related increase for both control and emphysematous groups, but there was no significant difference in the

Table 1. *Sarcomere number and connective tissue concentration in stimulated and immobilised rabbit soleus muscles*

	Group I Control	Group II Immobilised (shortened)	Group III Immobilised (shortened) & stimulated	Group IV Immobilised (lengthened)	Group V Stimulated only
No. of serial sarcomeres ($n = 30$)	8760∓160	5421∓184**	4639∓201**	9872∓230**	6374∓201**
Connective tissue (%)† $n \simeq 50$	5·4∓0·8	7·2∓1·1	5·5∓0·7 N.S.	4·9∓0·7 N.S.	5·1∓0·9 N.S.

Values are mean∓S.E.
* Significantly different from control ($P < 0.01$).
** Significantly different from control ($P < 0.001$).
† Percentage of muscle tissue profile area that stained for connective tissue.

Table 2. *Sarcomere number and connective tissue concentration in diaphragm muscle from emphysematous hamsters*

	6 weeks post-treatment		6 months pre-treatment	
	Control	Emphysema	Control	Emphysema
Sarcomere number ($n = 30$)	6160∓201	5620∓127*	6623∓164	5442∓138*
Connective tissue (%) † $n \simeq 50$	7·0∓1·4	7·8∓1·6 N.S.	10·4∓1·5	12·1∓1·9 N.S.

* Significantly different from control ($P < 0.3$).
** Significantly different from control ($P < 0.001$).
Values are mean∓S.E.
† Percentage muscle tissue profile area that stained for connective tissue.

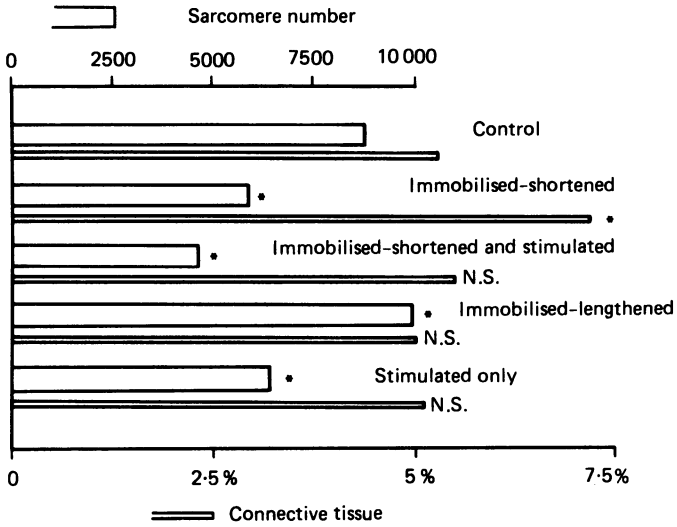

Fig. 1. The proportion of connective tissue and the number of serial sarcomeres in muscle immobilised in shortened and lengthened positions, with and without electrical stimulation.

proportion of connective tissue between the emphysematous and control groups at either age (Table 2).

DISCUSSION

When muscles were immobilised in the shortened position, there was, as in immobilised mouse muscle, a loss of serial sarcomeres and an increase in the concentration of connective tissue. However, when the immobilisation was combined with stimulation, connective tissue build-up was prevented implying that lack of contractile activity is an important factor in connective tissue accumulation.

No increase in the proportion of connective tissue occurred when muscles were immobilised in an extended position. This could be due to the mechanical effects of stretch. However, it has been shown for the immobilised soleus muscles of the rat that, whereas in the shortened position EMG activity falls rapidly, in the stretched position resting EMG activity remains at normal levels (Hnik *et al.* 1985). Thus it is possible that it is again neural activation which prevents the connective tissue build-up.

After 7 days of continuous stimulation serial sarcomere number was reduced. This is probably due to the fact that stimulation caused the limb to be held in a more dorsiflexed position than normal, i.e. the muscle was working at a reduced length as in the case of the diaphragm of the emphysematous hamsters. However, in neither the continuously stimulated rabbit soleus muscle nor in the emphysematous hamster diaphragm muscle was sarcomere loss accompanied by connective tissue proliferation. These results thus underline the role of contractile activity in maintaining normal connective tissue proportions. The observation of an unaltered pattern of connective tissue in the diaphragm muscle of emphysematous hamsters is in accordance with the results of Supinski & Kelsen (1982) who, in a physiological study of muscles from emphysematous hamsters, showed that despite differences in position and range, the passive tension–length curves of the diaphragms from emphysematous and control groups were identical when expressed as a function of sarcomere length.

The results of the present experiments thus provide further evidence of the correlation between the connective tissue component of a muscle and its compliance.

The experiments described here show that the loss of sarcomeres which occurs when a muscle is prevented from being stretched throughout its normal excursion is not always accompanied by an increase in the proportion of connective tissue and indicate that the reduced compliance which occurs in inactive muscles can be prevented by either passive stretch or active stimulation. These results have important implications for the treatment of many disabling conditions and also suggest that lack of activity may play a role in the accumulation of connective tissue in ageing muscle (Alnaqeeb, Al Zaid & Goldspink, 1984). Experiments are under way to determine whether short periods of stretch or short periods of stimulation are equally effective in preventing connective tissue accumulation in disused muscle.

SUMMARY

The loss of serial sarcomeres which results when muscles are immobilised in a shortened position is accompanied by an increase in the proportion of collagen and an increased muscle stiffness. In order to determine whether it is lack of stretch or lack of contractile activity which is the main factor involved in these changes experiments were carried out using different combinations of immobilisation and electrical stimulation. It was found that the connective tissue accumulation that occurs in inactive muscles can be prevented either by passive stretch or by active stimulation. It was also shown that in muscle that is working over a reduced range there is, as in muscle immobilised in the shortened position, a reduction in serial sarcomeres. In this case, however, there is no concomitant increase in connective tissue, again indicating that contractile activity is important for the maintenance of normal muscle compliance.

This work was supported by the Medical Research Council in Great Britain, the Veterans Administration Research Service and NIH grant 1 RO1 AG-04627-01A1 in the U.S.A. The authors thank Mrs A. Murtagh for her expert technical assistance and Mr P. Marshall who designed the computer programme for the image analysis.

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