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EFFECT OF ACTIVITY ON PERFORMANCE AND MORPHOLOGY IN ISCHAEMIC RAT SLOW MUSCLES

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Summary

Muscle performance and structure was studied in rat soleus muscle with limited blood supply in combination with chronic muscle stimulation. Blood supply to the lower leg was restricted by ligation of the common iliac artery, electrodes were implanted in the vicinity of the sciatic nerve and ankle flexors were denervated. Three days later, soleus and gastrocnemius muscles were stimulated at 4 Hz four times a day for a period of 20 min with 2 h intervals between stimulations; this procedure was continued for 4 days. Muscle performance, histochemistry and ultrastructure were studied on the eighth day after operation in these muscles and in ischaemic unstimulated muscles with denervated ankle flexors. Both were compared with control animals. Muscles with limited blood supply developed less isometric twitch tension than control muscles (peak twitch tension in ischaemic muscle was $60.3\pm4.8\,g\,g^{-1}$ muscle, mean±S.E.M., compared to $79.7\pm6.9\,g\,g^{-1}$ in control muscle; tensions after 5 min contraction were $54.5\pm5.5\,g\,g^{-1}$ in ischaemic muscle compared to $70.6\pm6\,g\,g^{-1}$ in controls). Stimulated muscles with limited blood supply had higher peak ($85\pm16.6\,g\,g^{-1}$) and final ($87\pm12\,g\,g^{-1}$) tensions, and also fatigued less than muscles with limited blood supply but no stimulation. Histochemical estimation of capillary density (by staining for alkaline phosphatase) and slow (SO) and fast (FOG) fibres (by myosin ATPase staining) revealed similar capillary to fibre ratios (2.5) and a similar proportion of FOG fibres (around 18%) in all muscles. The proportion of glycogen-depleted fibres (estimated from the periodic acid Schiff reaction, PAS) in muscles removed from animals 10 min after a 5 min period of isometric twitches was significantly lower in ischaemic muscles (45.1±1.9%) than in control (80.5±1.5%) or chronically stimulated ischaemic muscles (67.3±4.0%). Electron microscopy showed disorganised myofibrils with Z-line streaming in 7.48±3.04% of fibres in muscles with limited blood supply. Swollen and degenerated mitochondria, dilated sarcoplasmic reticulum and areas of disrupted sarcolemma were also observed. Stimulated ligated muscles showed a significantly lower proportion of fibres with disorganised filaments (0.65±0.32%) and other signs of damage were much less frequent. The

Key words: chronic stimulation, fatigue, ultrastructure.

reduced damage and improved performance of chronically stimulated slow muscle may be the result of improved microcirculation, preventing accumulation of lactate.

Introduction

Limitation of blood supply leads inevitably to decreased muscle performance and eventually to muscle fibre damage. These changes are more severe in muscles with predominantly oxidative metabolism. Thus Karpati *et al.* (1974) described a more extensive destruction of muscle fibres in soleus than in gastrocnemius in rats with a ligated abdominal aorta. Harriman (1977) found a greater proportion of necrotic fibres in soleus than in gastrocnemius after embolisation of the femoral artery in rabbits and Janda *et al.* (1972) observed a decrease in malate dehydrogenase activity only in the oxidative, but not in the glycolytic, part of tibialis anterior muscles in rats with limited blood supply.

Fibre damage was also described in chronic overload induced either by intensive exercise (Salminen *et al.* 1984; Salminen and Kiniström, 1985) or by extirpation of synergists (Roy *et al.* 1985). Increased activity of fast glycolytic fibres induced by chronic electrical stimulation also resulted in fibre damage (Maier *et al.* 1986; Maier and Pette, 1987), and this was greatly accentuated when blood flow to such muscles was inadequate. Hudlicka *et al.* (1988) described centrally located nuclei, Z-line streaming and loss of weight in rat fast skeletal muscles with limited blood supply, electrically stimulated for a total of 6 h a day for 9–12 days. Thus, both limitation of blood supply and exaggerated physical activity can result in muscle fibre damage.

Since exercise is, so far, the most extensively used treatment of peripheral vascular diseases, it is important to ascertain the balance between its beneficial and detrimental effects on muscle structure and performance. A mild regime of electrical stimulation of both fast and slow muscles with limited blood supply used by Elander *et al.* (1985) resulted in increased activity of oxidative enzymes, increased glycogen and decreased ATP content. Its effect on muscle performance or morphology was, however, not studied.

The purpose of this work was to ascertain whether a regime of muscle stimulation similar to that used by Elander *et al.* (1985) could improve muscle tension and fatiguability, and whether it would diminish the detrimental changes observed in slow muscles with high oxidative metabolism after limitation of blood supply. Preliminary results of this study have been published (Corsi *et al.* 1988).

Materials and methods

Experiments were performed on three groups of Sprague–Dawley rats, 370–450 g body mass. Four rats were used as controls without any intervention. Unilateral ligation of the right common iliac artery was performed in seven rats. Another group of seven rats had a ligated common iliac artery and underwent chronic stimulation of the ipsilateral soleus muscle.

Surgical procedures were performed under halothane anaesthesia and aseptic conditions. The right common iliac artery was ligated immediately below the bifurcation of the aorta. The lateral popliteal nerve, innervating ankle flexors (tibialis anterior, extensor digitorum longus and peroneal muscles), was sectioned to limit chronic stimulation to triceps surae. The electrodes, made of multi-stranded stainless-steel, Teflon-coated wire, were implanted in the vicinity of the sciatic nerve in the lower one-third of the thigh. They were led under the skin towards the back of the animals and soldered to a special socket which was sutured to the skin. Stimulation started 3 days after surgery. The animals were connected to a Grass S8 stimulator four times a day for 20 min with 2 h intervals between stimulations. This regime was established on the basis of a glycogen depletion and repletion experiment that showed complete repletion in muscles with intact blood supply and in slow fibres in muscles with limited blood supply. Stimulation parameters were 0.3 ms pulse duration, 4 Hz and a voltage (up to 5 V) that produced palpable contractions of ankle extensors without causing distress to the animals. Acute experiments were carried out approximately 14 h after the last stimulation, on the eighth day after ligation of the iliac artery. Animals with limited blood supply (and denervated ankle flexors) but not stimulated were also used in acute experiments on day 8 after the operation.

Acute experiments were performed under sodium pentobarbitone anaesthaesia (50 mg kg^{-1} intraperitoneally). The jugular vein was cannulated to supplement anaesthesia as required. The Achilles tendon on both sides was dissected free. Tendons of the gastrocnemius and plantaris muscles were cut and diverted, and the soleus tendon was connected to a strain gauge (25 g, Ether Ltd, UK) to record tension on a chart recorder and a dual beam oscilloscope. The animals were positioned on their ventral surface on a Perspex board fixed to the operation table. The hindlimbs were fixed to this board by a special holder and clamps on the paws that allowed recording of isometric tension without producing trauma to the limbs. Gastrocnemius and plantaris muscles were denervated and bipolar electrodes were placed on the popliteal nerve to induce isometric contractions after adjustment of the length of the muscle to give maximal tension. Tension was measured during a single twitch to assess contraction and half-relaxation time. This was followed by isometric twitches for 5 min at 4 Hz and finally 5 s tetanic contraction at 20 Hz.

Muscles were then quickly taken out and a cross-section about 5 mm thick from the mid-belly was frozen in isopentane precooled in liquid nitrogen. Serial sections 12 μm thick were cut on a cryostat and subsequently stained for succinate dehydrogenase (Nachlas *et al.* 1957) and myosin ATPase (Guth and Samaha, 1970) to identify muscle fibre types, and for alkaline phosphatase (Ziada *et al.* 1984) to identify capillaries; periodic acid Schiff reaction (PAS) was used to estimate the content of glycogen (Bancroft and Stevens, 1982). Serial samples were also fixed in 2.5 % glutaraldehyde in phosphate buffer, postfixed in 1 % osmium and embedded in Epon. Semithin sections were examined by light microscopy after Toluidine Blue staining. Ultrathin sections were stained with uranyl acetate and lead citrate

for transmission electron microscopy. To assess fibre damage, at least 200 fibres in each muscle were analysed in several cross-sections by light microscopy (a total of more than 8000 fibres in the whole study), and at least 80 fibres per muscle (more than 3300 fibres in all) were analysed in cross-sections under the electron microscope.

All values are given as means±S.E.M. Statistical analysis was performed using paired or unpaired Student's *t*-test in the assessment of muscle performance.

Results

Muscle performance

An original record of muscle tension is presented in Fig. 1. Both contralateral (normal) muscles showed a tension record typical of a slow muscle with a negative staircase. The tension in the muscle with limited blood supply was smaller, and the time-to-peak and relaxation time both indicated that the muscle was slower. Chronically stimulated muscle with limited blood supply showed higher tension, an initial negative staircase followed by a gradual increase in tension and a contraction speed similar to those of the contralateral muscle.

Table 1 summarizes the data on contraction and half-relaxation times. Although the average value for the contraction times was slightly higher in muscles with limited blood supply, the difference was not significant when compared to control muscles. However, muscles contralateral to the ligated ones showed a significantly shorter contraction and half-relaxation time when compared to either control or ischaemic muscles. This might be due to an increased used of the contralateral leg. Stimulated muscles with limited blood supply – as well as their contralateral muscles – had contraction and half-relaxation times similar to controls.

Muscles with limited blood supply developed less tension both at the beginning ($60.3\pm4.8\,g\,g^{-1}$) and at the end ($54\pm5.5\,g\,g^{-1}$) of the period of isometric twitches than either controls (79.7 ± 6.9 and $70.6\pm6.04\,g\,g^{-1}$, respectively) or stimulated muscles with limited blood supply (84.9 ± 16.6 and $87.1\pm12.1\,g\,g^{-1}$, respectively)

Table 1. *Time-to-peak and half-relaxation time in rat soleus: control animals, animals with unilateral (right) ligation of the common iliac artery, animals with ligated iliac artery and stimulated soleus*

		Ligated		Stimulated, ligated	
	Control	*C*	*E*	*C*	*E*
Time-to-peak (ms)	80.6±7.3 (7)	60.2±3.6* (6)	89.3±9.7 (6)	76.1±5.2 (8)	80.4±7.2 (7)
Half-relaxation time (ms)	77.7±10.4 (7)	54.3±5.7* (6)	84.8±6.7 (6)	75.6±6.5 (8)	78.2±11.1 (7)

C, contralateral muscle; *E*, experimental muscle.
Number of muscles in parentheses.
* $P<0.05$ compared with control and contralateral to experimental muscle.

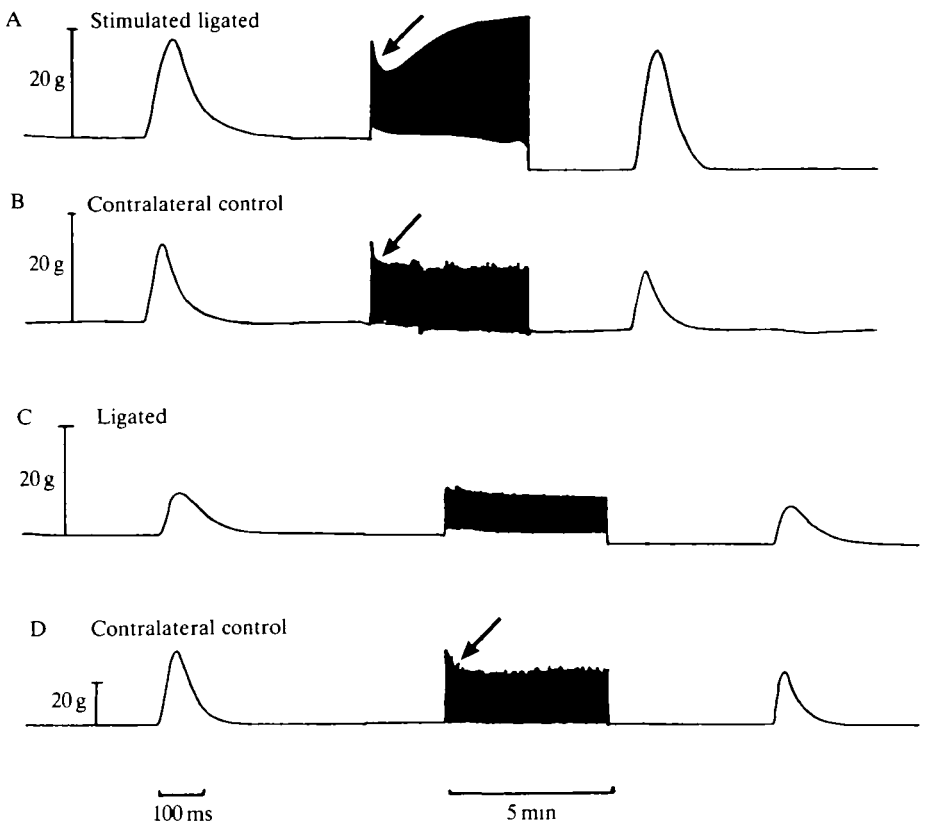


Fig. 1. Record of muscle tension during twitch contractions. The initial and final single twitch were recorded at high paper speed and traces were made at the same time from the oscilloscope screen to evaluate contraction and half-relaxation times. Muscles were contracting at 4 Hz during the 5 min period. (A) Tension recorded in stimulated ischaemic muscle; (B) in the contralateral muscle; (C) in ischaemic muscle; and (D) in the contralateral muscle. Arrows indicate negative staircase.

(Fig. 2). Stimulated ischaemic muscles actually showed an increase in tension during the course of contraction (Fig. 1). Tetanic tension was $558\pm66\,g\,g^{-1}$ muscle in control, $324\pm42\,g\,g^{-1}$ in ischaemic ($P<0.01$) and $501\pm47\,g\,g^{-1}$ in stimulated ischaemic muscles. Thus, limitation of blood supply resulted in a lower peak tension and appearance of fatigue normally not present in slow muscles. Stimulation of ischaemic muscles improved both maximal tension and resistance to fatigue.

Muscle mass

Muscle masses were expressed as a percentage of body mass since this enabled a

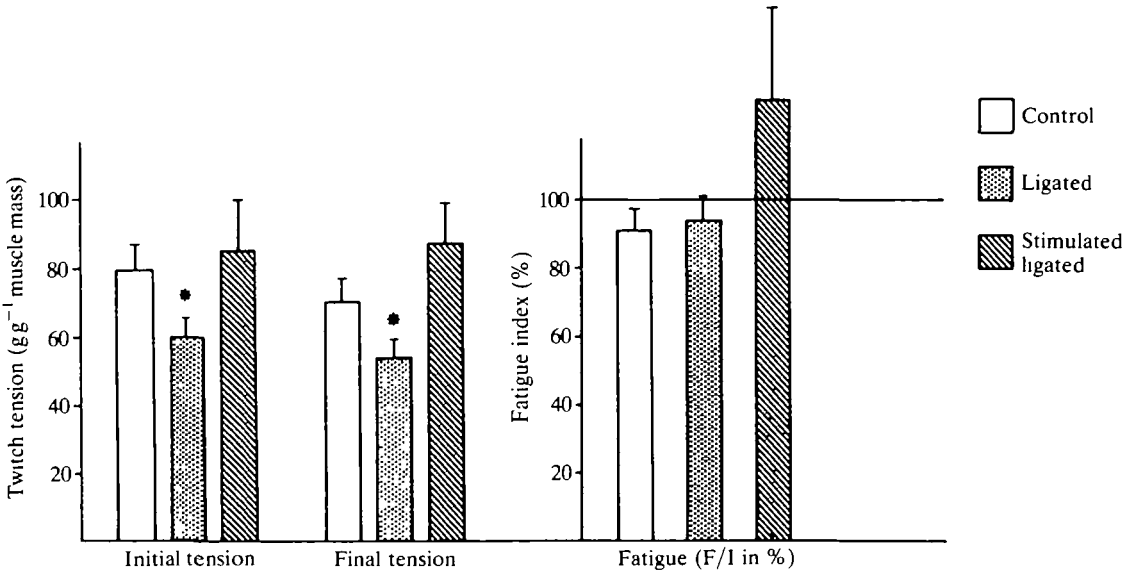


Fig. 2. Twitch tension (expressed in g g^{-1} muscle mass) in control, ischaemic (ligated) and ischaemic stimulated muscles. Final tension was measured at the end of a 5 min contraction period at 4 Hz. The fatigue index (FI) was calculated by dividing the final tension (F) by the initial tension (I) (which is represented as 100 %). * Significantly different from control, $P<0.05$ ($N=13$). Values are mean+S.E.

better comparison among groups. This ratio was 0.534±0.007 mg muscle g^{-1} body mass in control animals with no difference between right and left muscles (Fig. 3). Animals with a limited blood supply had heavier muscles in the contralateral leg (0.64±0.01 mg g^{-1}, significantly different from controls, $P<0.001$), whereas the affected muscle had a similar mass to controls – possibly indicating an increased usage of the intact leg. Stimulated ligated muscles were slightly, but not significantly, lighter than control muscles (0.48±0.02 mg g^{-1}) and significantly lighter than the contralateral muscles (whose mass, 0.56±0.03 mg g^{-1}, was not significantly different from controls).

Fibre types, glycogen depletion and capillary supply

Rat soleus has a population of oxidative fibres, the majority being slow and a small proportion fast. This proportion was similar in all muscles, representing 17.3±2.5% in ligated and stimulated ligated muscles and 18.6±2.1 % in control muscles. Capillary supply, evaluated as capillary to fibre ratio, was also similar in all muscles: 2.50±0.06 in ischaemic, 2.56±0.07 in stimulated ischaemic muscles and 2.46±0.05 and 2.54±0.06 in the respective contralateral muscles.

Staining for glycogen showed a great difference in the proportion of darkly stained (glycogen-containing) and unstained or lightly stained (glycogen-depleted) fibres. Contralateral muscles in both experimental groups had a high proportion of glycogen-depleted fibres 10 min after the end of the 5 min period of isometric

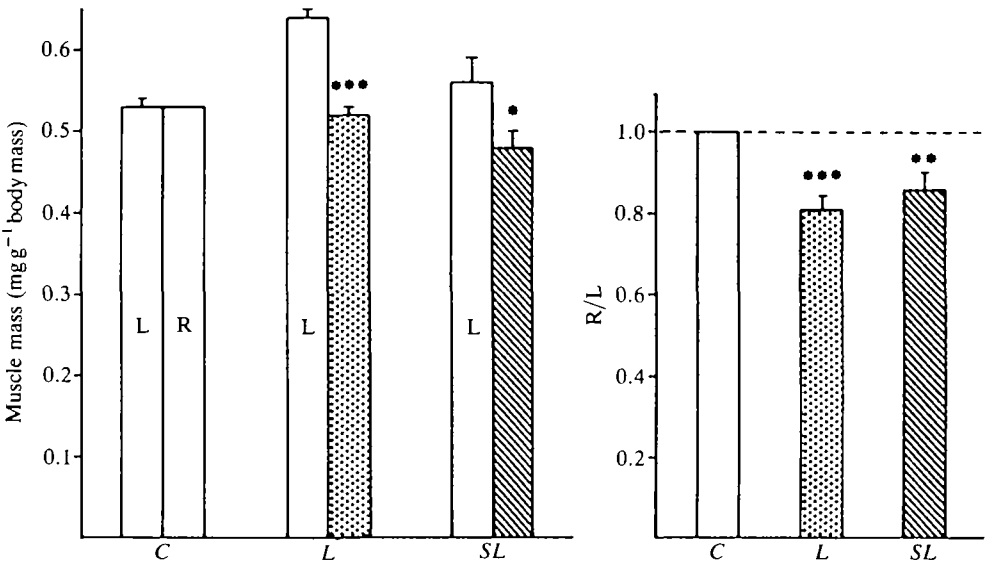


Fig. 3. Muscle masses (expressed as ratio of muscle to body mass in mg g^{-1}) in control animals (*C*), animals with ligated right common iliac artery (*L*) and animals with ligated common iliac artery and stimulated soleus (*SL*). L, left; R, right (experimental) muscle. * $P<0.05$; ** $P<0.01$; *** $P<0.001$ compared with the contralateral muscle. Values are mean+S.E. ($N=7$). The stippling and cross-hatching correspond to Fig. 2.

twitches, 90.4±3.0% in animals with a ligated iliac artery and 80.5±1.5% in the stimulated group. Ligated muscles had a significantly lower proportion of glycogen-depleted fibres (45.1±9.9%, $P<0.02$) than control muscles, indicating a relatively small usage of either muscle fibres or glycogen during contractions. The proportion of glycogen-depleted fibres in stimulated muscles with limited blood supply was significantly higher than in ischaemic and significantly lower than in control muscles (67.3±4.0, $P<0.05$). However, there was no correlation between the proportion of glycogen-depleted fibres and muscle tension in any of the groups studied.

Light and electron microscopy

Light microscopy

The observations were made on a sample of 4402 fibres from ischaemic muscles, 1514 fibres from stimulated ischaemic muscles and 2256 fibres from contralateral muscles. In cross-sections some fibres, mostly smaller than normal, stained weakly with Toluidine Blue because of defective and disorganised contractile elements: denser areas, corresponding to groups of filaments, were interspersed randomly among homogeneous or granular areas: centrally located nuclei could occasionally be seen (Fig. 4). Longitudinal sections showed that the changes were focal, involving only parts of the fibres. The proportion of abnormal fibres was

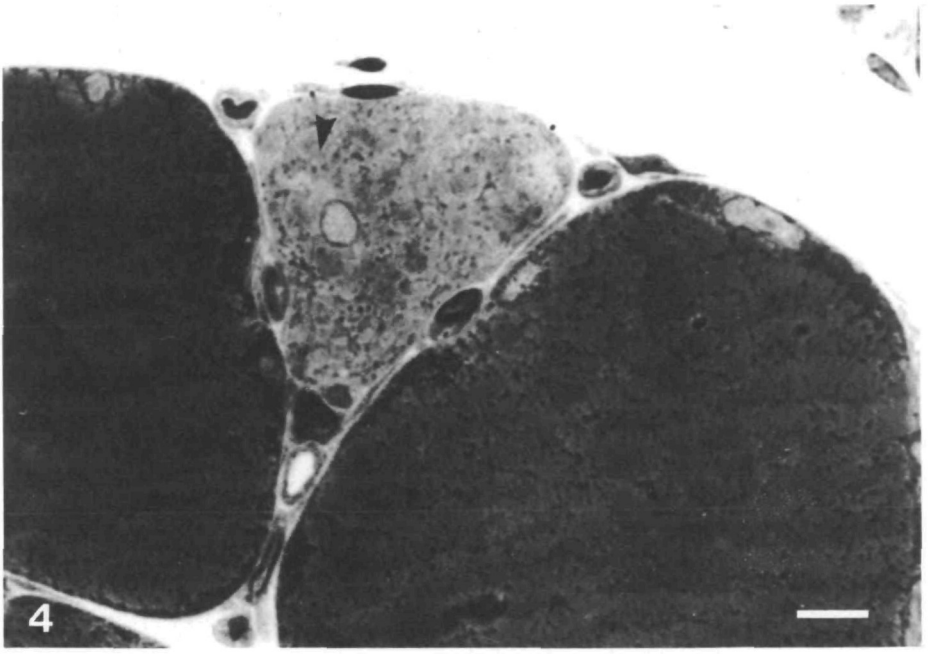


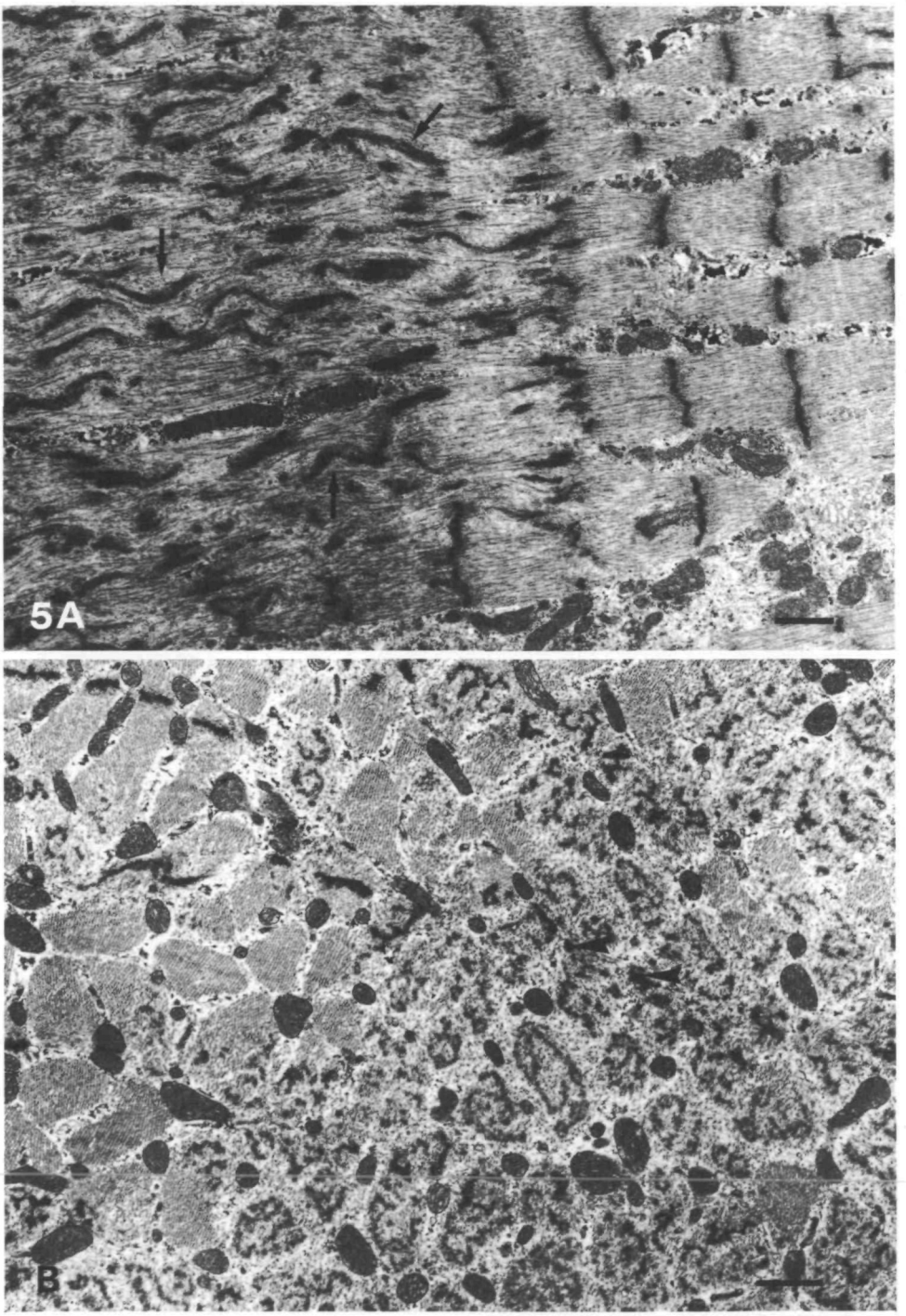
Fig. 4. Transverse section of a ligated soleus. The cytoplasm of a small-sized fibre is not homogeneous because of the presence of a pale, apparently structureless material (arrowhead). Scale bar, 10 μm.

4.0±0.9% in muscles with limited blood supply, 1.4±0.3% in stimulated ischaemic muscles and 0.52±0.19% in contralateral muscles.

Electron microscopy

The data were based on a sample of 1382 fibres from ischaemic muscles, 1020 fibres from stimulated ischaemic muscles and 237 fibres from contralateral muscles. The most prominent alteration apparent in the electron microscope was focal disarray of myofibrils with condensation of myofilaments, i.e. the change that is generally referred to as 'streaming of the Z-lines' (Fig. 5). Mitochondria with sparse cristae and autophagic vacuoles were found in some muscle fibres. Signs of regenerative activity in the form of abundant rough endoplasmic reticulum and free ribosomes were often associated with signs of degeneration in the same fibre (Fig. 6). Damage was less frequent in ischaemic muscles that had been chronically stimulated than in unstimulated muscles with limited blood supply. The pro-

Fig. 5. (A) Longitudinal section of ligated soleus. The elements of several sarcomeres are affected by the change usually referred to as 'streaming' of the Z-line: electron-dense material (arrows) replaces part of the Z-lines and of the A-bands. The right side of the fibre shows normal structure. Scale bar, 1 μm. (B) Transverse sections of a ligated soleus. Normal structure is shown in the upper left corner. Electron-dense material replaces the elements of several myofibrils. Note that the myofibrils are widely spaced with large numbers of granules between them (arrowheads). Scale bar, 1 μm.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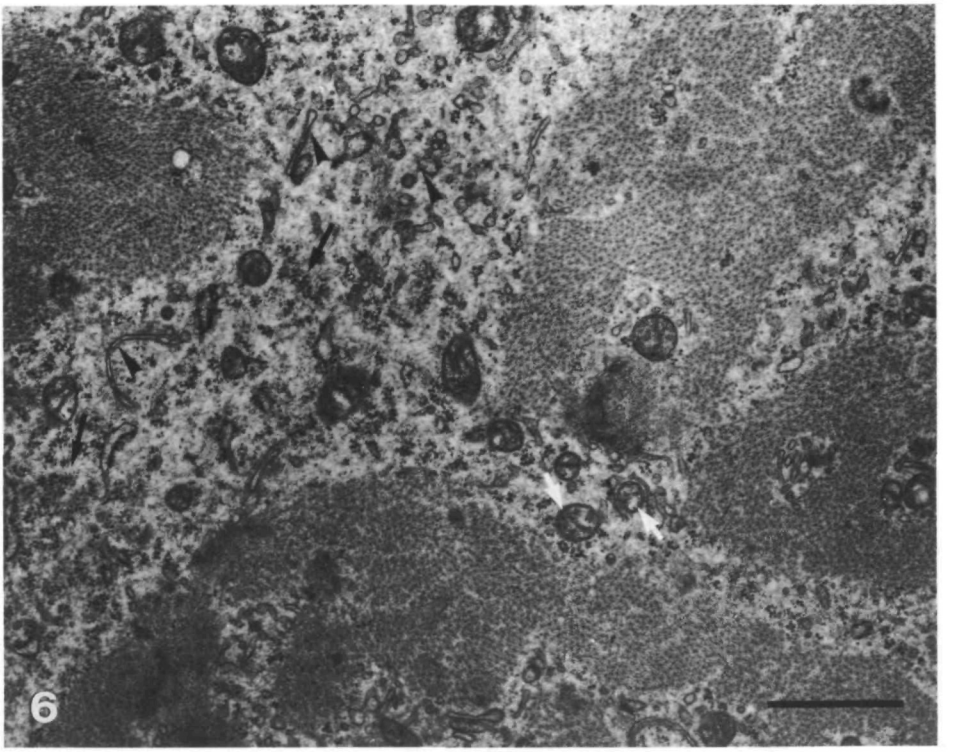

Fig. 6. Part of regenerative cell in a ligated soleus. Note abundant ribosomes (arrows), tubules and vesicles (arrowheads) of the sarcoplasmic reticulum and mitochondria among the myofibrils. Mitochondria with sparse cristae and vacuoles are marked by white arrows. Scale bar, 1 μm.

portion of fibres with disorganised myofibrils was 0.71±0.34 % in control muscles, 7.48±3.04 % in ischaemic and 0.65±0.32 % in stimulated ischaemic muscles. Although it is impossible to follow the longitudinal extension of disorganised myofibrils, the considerably higher incidence of fibres with abnormal ultrastructure, together with the increased proportion of abnormal fibres shown by light microscopy in ischaemic muscles, and the reversal of values found in control muscles by chronic stimulation, indicate strongly that mild stimulation of ischaemic muscles can prevent structural damage.

Discussion

The experiments confirmed previous findings (Karpati *et al.* 1974; Harriman, 1977) of muscle fibre damage in ischaemic oxidative muscles. This was demonstrated by light and electron microscopy and was also reflected in muscle performance: ischaemic muscles developed lower twitch and tetanic tension than control ones and fatigued more.

Elander *et al.* (1985) showed that stimulation of ischaemic fast or slow muscles -

using a regime similar to that applied in the present experiments – increased the activity of oxidative enzymes and glycogen content, but oxygen and glucose consumption were unchanged. They did not evalutate the effect of the stimulation on muscle performance. The present findings showed quite clearly a considerable improvement of both peak tension and resistance to fatigue in stimulated soleus. A similar improvement was found in chronically stimulated fast muscles (Hudlicka and Price, 1986), where it could have been due to an increased proportion of oxidative fibres and increased volume density of mitochondria (Hoppeler *et al.* 1986). The composition of soleus (100% oxidative and 80% slow fibres) was changed neither by ligation of the iliac artery nor by stimulation of ischaemic muscles. Elander *et al.* (1985) found higher activities of cytochrome oxidase and citrate synthase in stimulated soleus with limited blood supply than in either control or ischaemic muscles, so it is possible that improved oxidative capacity could contribute to improved performance achieved by stimulation. It is, however, also conceivable that chronic stimulation may have improved the microcirculation of the muscle.

The soleus is a muscle with an extensive capillary supply (Romanul, 1965). Although the capillary to fibre ratio was similar in control, ischaemic and stimulated ischaemic muscles, it is possible that the actual perfusion of capillaries was different. Dawson and Hudlicka (1989) showed a geater intermittency of red blood cell flow in capillaries in solei with limited blood supply than in control ones. Using vascular casts, Hudlicka and Torres (1990) showed that 1 week after ligation of the common iliac artery, there was a decrease in the size of the whole vascular bed in soleus to only 40% of the control level. This was linked with a considerable decrease in perfusion pressure (Dawson, 1989). Chronic stimulation of hindlimb muscles significantly improved the formation of collateral circulation (J. M. Dawson and O. Hudlicka, unpublished results) and thus it may be assumed that it would also normalise capillary perfusion.

Chronic stimulation also resulted in a significant decrease in the proportion of damaged muscle fibres found in ischaemic muscles. The damage due to ischaemia could be explained by accumulation of lactate, which would result in a decrease in pH and activation of lysosomal enzymes – a mechanism that was suggested to be involved in muscle fibre damage after strenuous exercise (Salminen *et al.* 1984) as well as in ischaemic rabbit muscles (Hagberg *et al.* 1985). Improvement of microcirculation by chronic stimulation would result in a faster removal of lactate and, consequently, a smaller percentage of damaged fibres. It is possible that it could also reduce the concentration of lysophospholipids produced by partial hydrolysis of phospholipids in the absence of an adequate oxygen supply, which was suggested as a possible factor involved in fibre damage by Franson *et al.* (1972).

Interestingly, the damage in ischaemic muscles was not extensive enough to affect muscle mass in relation to body mass. However, the observation that the contralateral muscles were heavier indicates that the contralateral leg was used more in animals with unilateral ligation of the common iliac artery than in normal

ones. A similar increase in the mass of contralateral muscles was observed in fast extensor digitorum longus and tibialis anterior of animals with a limited blood supply and which did not show any structural damage (Hoppeler *et al.* 1987). In our experiments improved performance of the stimulated ischaemic soleus obviously enabled the animals to rely less on the contralateral leg and this was reflected in contralateral muscle masses similar to controls.

Chronic stimulation, albeit on a limited scale, also resulted in a striking difference in the proportion of glycogen-depleted fibres in comparison with control and ischaemic muscles. All muscles were removed approximately 10 min after the end of the period of contractions, i.e. at a time when little if any glycogen could have been resynthesized (S. Egginton and O. Hudlicka, unpublished results). The high proportion of glycogen-depeleted fibres in contralateral muscles at this time indicates that glycogen was used as the main source of energy. Ischaemic muscles had less than half, and stimulated ischaemic muscles about 70 %, of all fibres glycogen-depleted. This could mean that their motor units were not recruited to the same extent as in normal muscles, that substrates other than glycogen were used or that glycogen breakdown or its metabolic pathway were impaired.

The first possibility could arise if ischaemia were to affect neuromuscular transmission. Acute ischaemia may reduce the amount of acetylcholine released from the nerve terminals (Desmedt and Borenstein, 1977). However, Veicstenas and Comande (1981) did not find any impairment of neuromuscular transmission in the course of recovery (up to 20 days) after 6 h of ischaemia in rabbit muscles. If the recruitment of motor units were to have been affected by ischaemia, or ischaemia and stimulation, there should be a correlation between the proportion of glycogen-depleted fibres and tension. This was not found in any of the groups studied. Stimulation of ischaemic muscles improved performance to such an extent that the final tension was similar to that of control muscles and yet the proportion of glycogen-depleted fibres was significantly smaller. Moreover, Armstrong and Peterson (1981) indicated that the number of motor units recruited in soleus in rats running on a treadmill with periodic occlusion of the common iliac artery was similar to that in control muscles.

Thus, the significantly lower final tension and smaller proportion of glycogen-depleted fibres in ischaemic compared to control muscles indicates a possible limitation of the use of glycogen as the main source of energy. Bass *et al.* (1979) found significantly lower activity of glycolytic enzymes in ischaemic soleus, which could result in decreased glycogen breakdown. Glycogenolysis could also be inhibited by an increased concentration of lactate (Helmreich *et al.* 1965). Although lactate can be oxidised in contracting normal muscle, particularly during intermittent exercise (Corsi *et al.* 1970), a decreased content of lactate dehydrogenase in ischaemic muscles (Presta *et al.* 1981) would inhibit or hinder this pathway. Stimulation of ischaemic soleus could result in a lower intramuscular lactate concentration due to improved microcirculation, but also possibly in utilisation of lactate for glycogen synthesis. Talmadge *et al.* (1989) demonstrated

that glycogen is synthesised from lactate in chronically active mouse gastrocnemius. Elander *et al.* (1985) found a slightly higher lactate concentration in stimulated ischaemic than in control muscles, but this was about three times lower than values reported in contracting soleus perfused at low flow (Walker *et al.* 1982). All these findings seem to indicate that ischaemic slow muscles use glycogen to a smaller extent than control muscles, and that chronic stimulation improves glycogen utilisation as an energy source.

Use of substrates other than glycogen for short contractions has been demonstrated in chronically stimulated fast muscles. Hudlicka *et al.* (1977) found an increased free fatty acid uptake, as well as increased activity of palmityl-CoA synthetase, and Reichmann *et al.* (1985) described a considerable increase in the activity of other enzymes involved in lipid utilisation. However, no data are available on usage of lipid in stimulated slow muscles with either normal or limited blood supply. Since lipid metabolism requires a greater amount of oxygen, and consequently a higher blood flow, it is unlikely that ischaemic muscles, whether stimulated or not, would use more lipids than muscles with a normal blood supply.

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