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An investigation of arterial insufficiency in rat hindlimb

An enzymic, mitochondrial and histological study

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1. A small animal model of arterial insufficiency has been used to investigate enzymic alterations in the gastrocnemius, plantaris and soleus muscles of the hypoperfused limb. 2. At 7 days after induction of arterial insufficiency by unilateral femoral artery ligation, there were significant increases in the maximal activities of hexokinase, phosphorylase and 6-phosphofructokinase, whereas the activities of citrate synthase and 2-oxoglutarate dehydrogenase remained unchanged. 3. Similar increases in hexokinase, phosphorylase and 6-phosphofructokinase were still apparent 8–10 weeks after unilateral artery ligation, although only hexokinase remained significantly higher than contralateral control values. No enhancement of oxidative enzyme activities was observed. 4. The results are discussed in relation to the conflicting findings reported by other groups investigating enzymic adaptations in patients with arterial insufficiency.

INTRODUCTION

There is no satisfactory medical treatment of peripheral vascular disease and the progress in non-surgical management is hampered by lack of basic knowledge of the pathophysiological changes which occur in the ischaemic limb (Clyne, 1980). In the accompanying paper (Challiss *et al.*, 1986) we have presented a small animal model of arterial insufficiency, induced by unilateral ligation and section of the femoral artery. In this model an amelioration in the metabolic response of rat gastrocnemius muscle during supra-maximal stimulation at 1 Hz following unilateral femoral artery section was related to a time-dependent improvement in muscle bloodflow (Challiss *et al.*, 1986).

In the human disease state a number of adaptations have been characterized in skeletal muscle. In patients with claudication, an enhanced oxidative capacity has been suggested by the increased maximal activities of several mitochondrial enzymes (Bylund *et al.*, 1976; Bylund-Fellenius *et al.*, 1981), although some studies have not reported this modification in oxidative capacity (Sokol & Kedzia, 1965; Henriksson *et al.*, 1980; Clyne *et al.*, 1985).

In the present study we have investigated the effect of unilateral femoral artery section in the rat on the activities of several enzymic reactions in the gastrocnemius, plantaris and soleus muscles, at an acute stage (7 days) and chronically at 8–10 weeks post section, thus enabling the acute results presented in this paper to be contrasted with those obtained by Elander *et al.* (1985*a*,*b*) and perhaps to compare the chronic data with the human claudication disease state. In addition we have used muscle histochemical technique to assess morphological damage to the ligated limb at 7 days.

MATERIALS AND METHODS

Animal preparation

Unilateral femoral artery ligation and section were performed as described previously (Challiss *et al.*, 1986). Following ether anaesthesia all animals had recovered within 20 min; in animals where the femoral artery was sectioned there were no differences noted in their walking ability. Moreover, operated animals were housed together with sham-operated control animals and appeared normally active. Rats were studied 7 days and 8–10 weeks after the operation. In all cases the relevant muscles of the contralateral limb were used to determine control values.

Maximal enzyme activity assays

Animals were killed by cervical dislocation and the three muscles (gastrocnemius, plantaris and soleus) of the ligated and contralateral limbs were rapidly dissected. Each muscle was weighed and minced with scissors and homogenized with a Polytron (setting 5, 2 × 20 s, 4 °C) in 9 vol. of 50 mM-triethanolamine buffer, pH 7.4, containing 1 mM-EDTA, 5 mM-$MgCl_2$ and 20 mM-2-mercaptoethanol. For the assay of citrate synthase and lactate dehydrogenase the original non-Triton-treated homogenates were diluted 50-fold in 100 mM-phosphate buffer, pH 7.4. Then Triton X-100 was added to the original triethanolamine homogenates (0.05% final concentration) and the homogenates were centrifuged at 1000 *g* (10 min, 4 °C). The Triton-treated supernatant was used for all enzyme assays except citrate synthase and lactate dehydrogenase.

Maximal enzyme activities were measured at 25 °C with a Perkin–Elmer Lamda 3 recording spectrophotometer as follows: glycogen phosphorylase (Bergmeyer, 1974), hexokinase (Zammit & Newsholme, 1976), 6-phosphofructokinase (Opie & Newsholme, 1967), lactate dehydrogenase (Shoubridge *et al.*, 1985), citrate synthase (Alp *et al.*, 1976) and 2-oxoglutarate dehydrogenase (Cooney *et al.*, 1981). Pyruvate and 2-oxoglutarate dehydrogenase activities were also determined in isolated mitochondria by the methods of Morgan-Hughes *et al.* (1982).

Histology

Rapidly dissected gastrocnemius muscles were quenched in isopentane pre-cooled in liquid N_2 and processed as described by Morgan-Hughes *et al.* (1977).

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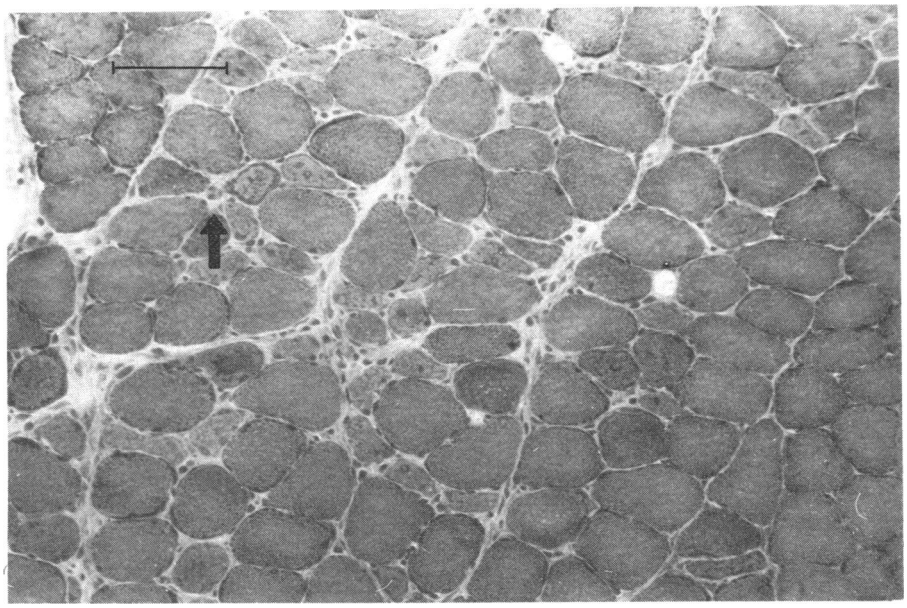

Fig. 1. Photomicrograph of the gastrocnemius of the rat 7 days after unilateral femoral artery ligation and section

The photomicrograph shows a section of the medial head of the gastrocnemius. There are diffuse sites of fibre necrosis (arrowed); the remainder of the section is normal. Scale bar represents 100 μm.

Preparation of skeletal muscle mitochondria

Two rats were killed by cervical dislocation and the gastrocnemius muscles rapidly dissected. Muscles from the ligated or contralateral limb were combined and mitochondria prepared using the trypsin (0.5 mg/g of tissue; Sigma type III) digestion method of Davies *et al.* (1981). After the final centrifugation step the mitochondrial pellet was resuspended in isolation medium (225 mM-mannitol/75 mM-sucrose/0.5 mM-EDTA/10 mM -Tris/HCl, pH 7.4) at a protein concentration of 6–10 mg/ml. Respiratory activities were measured at 25 °C as described by Morgan-Hughes *et al.* (1982).

Materials

All biochemical reagents and enzymes were obtained from either Boehringer Mannheim (Lewes, E. Sussex, U.K.) or Sigma (Poole, Dorset, U.K.). Protein concentration was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as a standard.

RESULTS

Histopathology

Freshly cut frozen muscle sections (10 μM) from the 7 day group were processed to demonstrate fibre types (ATPase reactions), mitochondrial location (succinic dehydrogenase) and morphology (haematoxylin and eosin). There were no differences noted in the fibre type composition of the ligated gastrocnemius muscles from their contralateral control muscles. There were no abnormal aggregates of mitochondria. There was no evidence of phagocytosis. However, some relatively mild abnormalities were observed; there was some evidence of diffuse fibre necrosis (Fig. 1). In more severe models of ischaemic muscle disease, induced by administration of dextran particles or by iliac ligation, marked morphological changes have been observed with scattered or localized areas of necrosis and phagocytosis as well as regenerating fibres (Hathaway *et al.*, 1969; Karpati *et al.*, 1974). Therefore section of the femoral artery produces only minor morphological alterations.

Table 1 Skeletal muscle weights of gastrocnemius, plantaris and soleus 7 days and 8–10 weeks after unilateral femoral artery ligation

Values are mean ± S.E.M. for five animals in each group.

	Muscle weight (g)			
	7 days		8–10 weeks	
Muscle	Control	Ligated	Control	Ligated
Gastrocnemius	1.13 ±0.03	1.07 ±003	1.42 ±0.01	1.33 ±0.05
Plantaris	0.226 ±0.009	0.224 ±0.010	0.270 ±0.010	0.260 ±0.010
Soleus	0.100 ±0.002	0.096 ±0.007	0.115 ±0.003	0.111 ±0.005

Enzymic adaptation

In the preceeding paper we noted a time-dependent improvement in the hyperaemic response during 1 Hz stimulation (Challiss *et al.*, 1986), following unilateral femoral artery section. Furthermore we suggested that enzymic alterations may have occurred during the time course of our study, and such findings are reported in this paper. Indeed, the hypothesis outlined by Bylund-Fellenius *et al.* (1981) that hypoperfusion and periods of transient hypoxia promote induction of aerobic enzyme synthesis has been tested for the rat in the present paper as well as recently by her group (Elander *et al.*, 1985*a*,*b*). The original observation that there was an enhanced oxidative capacity was based on human muscle biopsy studies from claudicants in which significant increases in

Table 2. Maximal enzyme activities in the gastrocnemius, plantaris and soleus muscles of rats 7 days and 8–10 weeks after unilateral femoral artery ligation and section

Femoral artery ligation of rats and measurement of maximal enzyme activities were performed as described in the Materials and methods section. Enzyme activities were measured in muscles of the ligated and non-ligated contralateral limb. Values are presented as means ± S.E.M. with the number of determinations given in parentheses. Statistical significance was determined by using Student's *t*-test (for paired observations) and is indicated as $*P < 0.05$, $**P < 0.01$. C, control; L, ligated; N.D., not determined.

		Activity (units/g wet wt.)					
		7 days			8–10 weeks		
Enzyme		Gastrocnemius	Plantaris	Soleus	Gastrocnemius	Plantaris	Soleus
Hexokinase	C	0.90±0.06 (5)	1.15±0.09 (5)	1.27±0.09 (5)	0.79±0.06 (5)	1.01±0.04 (10)	0.90±0.07 (5)
	L	1.47±0.19 (5)*	1.71±0.11 (5)*	2.07±0.15 (5)**	0.89±0.07 (5)	1.13±0.04 (10)*	1.18±0.10 (5)**
Phosphorylase	C	40.7±3.0 (10)	48.8±3.7 (7)	6.6±0.3 (5)	49.8±2.9 (5)	49.2±2.4 (5)	4.7±0.7 (5)
	L	48.9±3.2 (10)**	55.8±3.7 (7)*	7.4±0.4 (5)*	54.3±3.5 (5)	52.8±4.1 (5)	4.5±0.6 (5)
6-Phospho-	C	37.9±2.1 (8)	N.D.	N.D.	56.2±2.5 (5)	52.8±2.9 (5)	10.1±0.5 (5)
fructokinase	L	46.2±2.0 (8)*	N.D.	N.D.	57.4±2.9 (5)	55.1±3.2 (5)	10.6±0.5 (5)
Lactate	C	459±33 (10)	513±14 (5)	111±4 (5)	402±29 (5)	512±21 (5)	99±7 (5)
dehydrogenase	L	446±35 (10)	549±12 (5)	96±4 (5)	407±24 (5)	501±13 (5)	100±8 (5)
Citrate	C	17.9±0.7 (14)	21.8±1.0 (5)	23.2±0.5 (5)	17.9±1.5 (5)	22.6±2.1 (5)	19.9±0.9 (5)
synthase	L	18.4±1.2 (14)	22.0±1.0 (5)	24.1±0.9 (5)	18.5±1.2 (5)	22.0±1.9 (5)	20.2±1.0 (5)
2-Oxoglutarate	C	1.41±0.19 (5)	1.50±0.12 (5)	1.26±0.16 (4)	1.80±0.19 (5)	1.83±0.16 (5)	0.97±0.07 (5)
dehydrogenase	L	1.39±0.13 (5)	1.55±0.13 (5)	1.35±0.08 (4)	1.69±0.07 (5)	1.86±0.10 (5)	1.02±0.10 (5)

the maximal rates of some oxidative enzymes were noted (Bylund *et al.*, 1976; Bylund-Fellenius *et al.*, 1981). However these findings were not confirmed by other workers (Sokol & Kedzia, 1965; Henriksson *et al.*, 1980; Clyne *et al.*, 1985).

The wet weights of the muscles studied are shown in Table 1 for animals at 7 days (body wt. 200–240 g) and at 8–10 weeks (body wt. 280–320 g) following unilateral femoral artery section. There was no evidence of muscle atrophy. The maximal rates are expressed as units/g wet wet. for the enzyme activities measured and are shown in Table 2. At 7 days after femoral artery section there were significant increases in the activities of phosphorylase, hexokinase and 6-phosphofructokinase, with lactate dehydrogenase remaining unchanged. These alterations were still apparent in the 8–10 week group, although only hexokinase remained statistically significantly different (Table 2). We also measured activities of two oxidative enzymes, citrate synthase and 2-oxoglutarate dehydrogenase, and these were similar to their mean control values at both 7 days and 8–10 weeks post ligation (Table 2). The specific mitochondrial activities of these two enzymes were also measured, together with pyruvate dehydrogenase (Table 3), at 7 days post operation; again no differences were noted. The respiratory activities of isolated mitochondria from ligated and contralateral limbs were also studied (Tables 3 and 4). The rates of oxygen uptake with the various substrates tested were not significantly different from their mean control values. The activity of cytochrome oxidase was tested by presenting ascorbate + tetramethyl phenylenediamine (TMPD) as the substrates, and since the specific and wet weight citrate synthase activities were similar in the control and ligated limbs respectively (Tables 2, 3 and 4) then one can assume that the activity of cytochrome oxidase (on a wet weight basis) would also be similar. Therefore we have noted no differences in the rates of oxidative enzymes. However, the maximal rates of phosphorylase, hexokinase and 6-phosphofructokinase were elevated 7 days after unilateral femoral artery section and this observation was still apparent 8-10 weeks later (Table 2).

DISCUSSION

Unilateral femoral artery section in the rat produced a relatively mild model of skeletal muscle hypoperfusion. There were no gross morphological abnormalities (Fig. 1), unlike the models reported by Hathaway *et al.* (1969) and Karpati *et al.* (1974). At a time when bloodflow to the gastrocnemius, plantaris and soleus muscles was reduced during stimulation at 1 Hz to 59%, 63% and 49% of their contralateral control values respectively (7 days after femoral artery ligation), we measured several 'marker' enzyme activities. The maximal rates of hexokinase, phosphorylase and 6-phosphofructokinase measured were significantly increased (Table 2), whereas the maximal rates of citrate synthase and 2-oxoglutarate dehydrogenase were similar to their respective control values (Table 2). A 2-fold increase in the maximal activity of hexokinase was reported in rat soleus muscle 14 days after iliac ligation and citrate synthase was significantly lower than control values (Bass *et al.*, 1979). When the bloodflow to the sectioned lower limb could be assumed to be normal during 1 Hz stimulation (i.e > 14 days; Challiss *et al.*, 1986), we repeated the enzymic study. Thus at 8–10 weeks after arterial section the increased activities of the glycolytic enzyme activities were still apparent, although only the increase in hexokinase remained statistically significant (Table 2). However, more importantly, there was no evidence of an enhanced oxidative capacity. Moreover, the respiratory activities of mitochondria isolated from animals 7 days and 10 weeks

post ligation were similar in the control and ligated gastrocnemius muscles (Tables 3 and 4).

Our data are in contrast with those reported in some human studies of arterial insufficiency (Bylund *et al.*, 1976; Bylund-Fellenius *et al.*, 1981) and other animal models of arterial ligation (Elander *et al.*, 1985*a*,*b*). In the human studies of Bylund-Fellenius *et al.* (1981) there were significant increases in the maximal rates of cytochrome oxidase, citrate synthase and hydroxylacyl-CoA dehydrogenase in the gastrocnemius muscle of claudicants. There were no differences in the activities of lactate dehydrogenase or 6-phosphofructokinase. However, in a similar study two groups have reported increased activities of lactate dehydrogenase, phosphorylase and pyruvate kinase, whereas citrate synthase activity remained at the control value or decreased significantly (Henriksson *et al.*, 1980; Clyne *et al.*, 1985). In addition, these latter two investigations confirmed the earlier histochemical observations of Sokol & Kedzia (1965). The reason for this apparent discrepancy remains unclear, although exercise or detraining effects may play some role.

Table 3. Respiratory and enzyme activities in mitochondria isolated from gastrocnemius muscle of rats 7 days after unilateral femoral artery ligation and section

Mitochondria were prepared from the gastrocnemius muscle of the ligated limb or from the contralateral control limb as described in the Materials and methods section. Values are means ± S.E.M. for four mitochondrial preparations (gastrocnemius muscles of two animals were taken for each preparation). State-3 respiration was produced by addition of 250 nmol of ADP and is given as nmol of O/min per mg of mitochondrial protein at 25 °C. The respiratory control ratio (R.C.R.) is the relative State-3/State-4 oxygen consumption rate. Abbreviation: TMPD, tetramethylphenylenediamine.

	Respiratory activities			
	Control limb		Ligated limb	
Substrate	State-3 rate	R.C.R.	State-3 rate	R.C.R.
5 mM-Pyruvate + 2.5 mM-L-malate	213 ± 5	6.3 ± 0.4	227 ± 17	8.6 ± 0.7
10 mM-2-Oxoglutarate + 2.5 mM-L-malate	231 ± 30	6.6 ± 1.1	202 ± 18	7.6 ± 1.1
40 μM-DL-Palmitoyl carnitine + 2.5 mM-L-malate	154 ± 38	5.1 ± 0.8	153 ± 25	5.6 ± 0.8
2 mM-L-Ascorbate + 50 μM-TMPD	296 ± 8	—	259 ± 15	—

	Enzyme activities (nmol/min per mg of mitochondrial protein) at 25 °C	
Enzyme	Control limb	Ligated limb
Pyruvate dehydrogenase*	104 ± 9	94 ± 5
Oxoglutarate dehydrogenase	194 ± 14	156 ± 7
Citrate synthase	1082 ± 78	1144 ± 66

* Basal activity of pyruvate dehydrogenase was measured.

Table 4. Respiratory activities in mitochondria isolated from gastrocnemius muscle of rats 8–10 weeks after unilateral femoral artery ligation and section

Mitochondria were prepared from the gastrocnemius muscle of the ligated limb or from the contralateral control limb as described in the Materials and methods section. Values are means ± S.E.M. for three mitochondrial preparations (each prepared by combining two gastrocnemius muscles). State-3 respiration was produced by addition of 250 nmol of ADP and is expressed as nmol of O/min per mg of mitochondrial protein at 25 °C. The respiratory control ratio (R.C.R.) is the relative State-3/State-4 oxygen consumption rate. Abbreviation: TMPD, tetramethylphenylenediamine.

	Respiratory activities			
	Control limb		Ligated limb	
Substrate	State-3 rate	R.C.R.	State-3 rate	R.C.R.
5 mM-Pyruvate + 2.5 mM-L-malate	185 ± 23	13.5 ± 1.9	192 ± 18	17.3 ± 1.9
10 mM-2-Oxoglutarate + 2.5 mM-L-malate	192 ± 5	7.3 ± 1.3	170 ± 20	12.7 ± 0.5
10 mM-DL-Succinate + 10 μM-rotenone	167 ± 16	6.5 ± 1.7	201 ± 25	6.2 ± 0.5
2 mM-L-Ascorbate + 50 μM-TMPD	347 ± 37	—	320 ± 44	—

In the animal model of hypoperfusion reported by Elander *et al.* (1985*a*,*b*) the site of arterial ligation was at the level of the iliac artery. This ligation was also coupled to chronic bilateral nerve stimulation. A marked reduction in muscle blood flow was noted; however the weight and sex of the rats used for enzyme and metabolite measurements (female, 200–240 g) were different from those of the animals used in the microsphere blood flow studies (male, 380–420 g). They observed a marked increment over and above the contralateral stimulated control limb in the apparent oxidative capacity of the soleus and extensor digitorum longus muscles. However they failed to measure these parameters in the muscles of non-stimulated arterially ligated rats. In our study, we have measured metabolite concentrations (Hayes *et al.*, 1985; Challiss *et al.*, 1986) and enzyme activities in normally active rats. Our model of arterial insufficiency results in the increased activities of the glycolytic enzymes (Table 2) and this may indeed be the metabolic response to hypoperfusion *per se* (Bass *et al.*, 1979). However, if the hypoperfused muscles are subjected to an additional stress, e.g. increased muscular activity (prescribed voluntary exercise in the claudicants or chronic nerve stimulation in the case of the rat model), then these two variables may act synergistically to increase the oxidative capacity of those muscles above that observed in their respective unaffected control. Moreover, endurance training following arterial section in the rat has demonstrated an enhanced oxidative capacity in the soleus and anterior tibial muscles relative to untrained sectioned animals (Janda *et al.*, 1972; Urbanova *et al.*, 1974).

Perhaps it is possible that vasodilation and the opening and development of the collateral circulation is dependent upon the site of the ligation, although it would seem improbable that hypoperfusion may signal the induction of two divergent enzyme pathways (i.e. anaerobic versus aerobic) which is dependent upon the site of the arterial ligation. It would seem logical that the signal is similar and that the other variable, chronic nerve stimulation, is the agent responsible for the difference. Since it is proven that endurance training or chronic nerve stimulation promote increases in oxidative enzyme capacity (Holloszy & Booth, 1976; Heilig & Pette, 1980), this effect may have dominated the situation in the iliac artery ligation study (Elander *et al.*, 1985*a*,*b*)

Therefore, our model provides a method of studying arterial insufficiency independent of induced muscular activity; moreover, it provides a protocol which causes an increase in the activity of certain enzymes (Table 2). In a situation where oxygen delivery was impaired it would be logical to increase ATP production via anaerobic glycolysis. In a study where short term energy supply was limited by depleting phosphocreatine concentration by replacement with a creatine analogue (β-guanidinopropionic acid), which is a poor substrate for the creatine phosphokinase reaction (Shoubridge & Radda, 1984), the metabolic adaptation was to increase the oxidative capacity (Shoubridge *et al.*, 1985). Under these conditions it has been suggested that the lowered phosphocreatine concentration ($< 10\%$ of the mean control value) 'signalled' increased expression of the oxidative enzymes (Shoubridge *et al.*, 1985). In the study by Elander *et al.* (1985*a*,*b*) chronic nerve stimulation would cause periods of reduced phosphocreatine concentration which would be more pronounced in the ligated limb both during stimulation and in recovery (see Challiss *et al.*, 1986), and this might prove sufficient to promote enhanced synthesis of the oxidative enzymes in the ligated limb over and above that observed in the control limb.

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