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Effects of torbafylline, pentoxifylline and buflomedil on vascularisation and fibre type of rat skeletal muscles subjected to limited blood supply

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1 Blood flow (measured by radio-labelled microspheres), fibre composition and capillary/fibre ratio were estimated in rat fast twitch skeletal muscles (tibialis anterior and extensor digitorum longus) five weeks after unilateral ligation of the common iliac artery in animals treated with either saline, torbafylline, pentoxifylline or buflomedil.

2 The resting blood flow was lower in muscles with limited blood supply than in their contralateral controls; this difference became statistically insignificant after treatment. Capillary/fibre ratio was similar in all muscles with either intact or limited blood supply and did not change after administration of any of the drugs.

3 The percentage of glycolytic fibres was not changed by ligation, but it decreased significantly in animals treated with torbafylline. This may improve performance in muscles with limited blood supply.

Introduction

Limitation of blood supply to skeletal muscles, whether acute or chronic, is almost inevitably linked with impaired muscle performance. Although some collateral circulation usually develops (Imparato *et al.*, 1975), which can satisfactorily meet the oxygen demand of muscles at rest, it is not sufficient to ensure adequate supply even during moderate exercise (Gaethgens *et al.*, 1976) and the result is intermittent claudication. Reconstructive surgery which normalises muscle metabolism (Pernow *et al.*, 1975), or exercise therapy, which is very beneficial (Larsen & Lassen, 1966; Zetterquist, 1970) is not always applicable. Consequently, there is a constant search for drugs which improve muscle blood flow and performance.

Close-arterial administration of adenosine 5'-triphosphate (ATP) increases blood flow in the occluded region (Schoop, 1956; hess, 1964), but it cannot be used routinely in the clinic. Blood flow after arterial thrombosis was increased, at least temporarily, by a shortlasting infusion of noradrenaline and this effect was attributed to the increased perfusion pressure (Dahn *et al.*, 1967). Bradykinin was without effect, or even impaired blood flow (Pernow & Zetterquist, 1967) in muscles with limited blood supply. Numerous data on pentoxifylline (Ward & Clissold, 1987) indicate that this drug improves microcirculation possibly by increasing the deformability of red blood cells. It is used routinely in patients with peripheral vascular diseases, but the data on the improvement of walking distance show great variability. Buflomedil was reported to improve blood flow in patients with peripheral vascular diseases both during acute and chronic administration (Perego *et al.*, 1982), but did not change it in hind-limbs of dogs with surgically limited vascular supply (Fujita *et al.*, 1984). It did increase oxygen tension, either by decreasing oxygen consumption (Sunder-Plassman *et al.*, 1981b) or by improving collateral circulation (Sunder-Plassman *et al.*, 1981a) but the data on its effect on muscle performance are variable (Clissold *et al.*, 1987).

Although muscle performance, measured mainly in terms of fatiguability in cases of peripheral vascular diseases, is related to blood supply, it can also be improved by a change in the composition of the muscle fibres. It is well known that muscles with a high proportion of oxidative fibres (type I or IIa) fatigue much less than those with a high proportion of glycolytic (type IIb) fibres (Burke *et al.*, 1971). Decrease in type I (slow oxidative) fibres was described in patients with muscle cramps (Telerman-Tuppet *et al.*, 1985); Clyne *et al.* (1982) did not find any change in the proportion of type I or IIa fibres, while Hammarsten *et al.*, (1980) described a higher proportion of type IIa fibres in patients who had intermittent claudication for at least one year. However, there are no data on the effect of drugs on fibre composition in muscles with limited blood supply.

The purpose of this study was to compare the long-term effect of several drugs, two of them (pentoxifylline, Hoechst, Werk Albert, and buflomedil, Abbot) routinely used in the clinic and one experimental drug (torbafylline, [7-ethoxymethyl-1-(5 hydroxy-5-methylhexyl)-3-methylxanthine], Gebert *et al.*, 1987; Okayayuz-Baklouti, 1988) on blood flow, fibre composition and capillary supply in skeletal muscles with limited blood supply.

Methods

Experiments were performed in four groups of male Sprague Dawley rats 480–550 g body weight. The right common iliac artery was ligated (under halothane anaesthesia and aseptic conditions) immediately below the bifurcation of the aorta in all animals. One group (8 animals) served as controls: four animals did not receive any treatment, four received 0.3 ml sterile saline solution intraperitoneally 3 times per day. The second group (7 animals) was injected following the same protocol with torbafylline 3 mg kg^{-1} body weight in 1% aqueous solution, the third group (6 animals) received 0.3 ml 1% (3 mg kg^{-1}) pentoxifylline and the fourth group (6 animals) 0.2 ml 1% (2 mg kg^{-1}) buflomedil. Treatment always started on the day after the operation, and the last injection was given the day before the final experiment, i.e. approximately 16 h before blood flow was estimated. All animals were treated for 5 weeks (7 days per week), with no obvious impairment of function (tested by the toe spreading reflex and walking) in the ligated leg under resting conditions. Animals on buflomedil showed a greater excitability after one week of treatment and thereafter.

Acute experiments were performed at the end of the treatment, under pentobarbitone anaesthesia. Blood flow at rest was measured using 15 μm microspheres labelled with ^{113}Stannum (Hudlická *et al.*, 1981) with injection into the left ventricle via the right carotid artery and withdrawal from one brachial artery. The contralateral brachial artery was cannulated to measure blood pressure. Samples of tibialis anterior

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(TA) and extensor digitorum longus (EDL) (whole cross section of the muscle in the mid-belly) were frozen in isopentane, precooled in liquid nitrogen. The rest of the muscles were used for blood flow estimations. The whole gastrocnemius muscle was also taken for blood flow measurements. To check adequate mixing of microspheres, blood flow was measured in the right and left kidney. The kidney flow (cortex) was $6.53 \pm 1.7\,ml\,g^{-1}\,min^{-1}$ in the control group, and was not affected by drug treatment. The difference between right and left kidney did not exceed 10%.

Sections (10 μm) from TA and EDL were cut on the cryostat, and stained for succinate dehydrogenase (Nachlas *et al.*, 1957) to show oxidative fibres, and for alkaline phosphatase using indoxyl-tetrazolium staining (Ziada *et al.*, 1984) to depict capillaries. In each muscle, 20 muscle bundles (300–400 muscle fibres) were counted for the number of muscle fibres and the number of capillaries, and the results were expressed as C/F ratio. In tibialis anterior, which is composed of a predominantly glycolytic superficial layer, and a predominantly oxidative core, counting was done separately in each part. Succinate dehydrogenase is present in minimal concentration in glycolytic fibres which appeared very pale. The percentage of these fibres was also counted in 20 bundles from each muscle, taking again the glycolytic and oxidative parts of the tibialis separately.

Student's *t* test (paired or unpaired, as appropriate) was used for statistical evaluation. Coefficient of variation (mean × 100)/s.d. was used to express variability of data.

Results

Body weight and muscle weight

All groups, with the exception of the animals treated with buflomedil, had similar body weights (Table 1). The slightly lower average body weight in animals on buflomedil was due to the fact that the animals were lighter at the beginning of the experiment. Similar increase in body weight (about 40 g) was observed in all groups over the period of five weeks. Tibialis anterior (TA) and extensor digitorum longus (EDL) muscles were significantly lighter in the ligated than in contralateral legs in control animals, and those treated with pentoxyfylline and buflomedil.

Blood pressure and blood flow

No significant differences among the groups were found in systolic, diastolic or mean blood pressure at rest (Table 2). Blood flow in gastrocnemius muscles did not seem to be affected by either drug treatment or the ligation of the common iliac artery. The average values of resting blood flow in contralateral TA and EDL muscles were higher in animals treated with torbafylline and pentoxifylline than in the untreated or the buflomedil-treated group, but the difference was not statistically significant using unpaired *t* test (Table 2). Similarly, long-term treatment with torbafylline and pentoxifylline slightly, but not significantly, increased resting flow in muscles with limited blood supply. Because of great variability of blood flow, particularly in torbafylline- and pentoxifylline-treated animals, flow in ligated muscles was calculated as a percentage of the flow in contralateral muscles, i.e. flow in the ligated leg was divided by flow in the contralateral leg. These values, although somewhat different from the proportions of means calculated from the data in Table 2, show more precisely the respective effect of individual drugs on the improvement of flow with respect to the contralateral muscles. Thus the mean of these values in ligated muscles represented $64.4 \pm 11.1\%$ of the value in the contralateral muscles in the untreated animals, being significantly ($P < 0.02$ against 1.0) lower. The relative decrease was smaller, $72.5 \pm 11.6\%$ ($P < 0.05$ against 1.0) in the torbafylline-treated group, and the values were not significantly different from 100% in the pentoxifylline ($73 \pm 13.8\%$, NS) and buflomedil ($85.7 \pm 19.5\%$, NS) groups, respectively. Thus, treatment with any of these drugs seemed to improve resting blood flow 5 weeks after the ligation of the common iliac artery. However, the lack of significant difference between the absolute values of flow in contralateral and ligated muscles, particularly in animals treated with pentoxifylline, was due to a tremendous variabil-

Table 1 Body weight (in g) and muscle weights (in g) of rats used in this study

Treatment	*Saline*	*Torbafylline*	*Pentoxifylline*	*Buflomedil*
Body weights	521 ± 8.7 (8)	536 ± 8.8 (7)	538 ± 4.0 (6)	507 ± 12 (6)
Muscle weights				
TA + EDL L	1.18 ± 0.01*	1.16 ± 0.06	1.12 ± 0.03*	0.98 ± 0.05**
C	1.25 ± 0.02	1.23 ± 0.03	1.27 ± 0.03	1.14 ± 0.05
Gastro L	2.79 ± 0.06	2.71 ± 0.11	2.86 ± 0.05	2.48 ± 0.04
C	2.85 ± 0.09	2.91 ± 0.08	2.82 ± 0.19	2.54 ± 0.05

Mean ± s.e.mean. Number of animals in parentheses.
L = ligated side, C = contralateral side, TA = tibialis anterior, EDL = extensor digitorum longus, Gastro = gastrocnemius.
* Significantly different from contralateral at $P < 0.005$.
** Significantly different from contralateral at $P < 0.05$.

Table 2 Mean blood pressure at rest (mmHg) and blood flow (ml $100\,g^{-1}$ × min) at rest and during contractions

Treatment	*Saline*	*Torbafylline*	*Pentoxifylline*	*Buflomedil*
BP systolic	139 ± 15	136 ± 11	128 ± 11	133 ± 5
diastolic	113 ± 13	112 ± 8	103 ± 9	108 ± 6
BP mean	124 ± 12.1 (8)	120.3 ± 7.4 (7)	111.0 ± 8.8 (6)	115.7 ± 4.6 (6)
Blood flow at rest				
TA + EDL L	2.54 ± 0.57 (6)*	3.99 ± 1.07 (7)	4.28 ± 1.36 (6)	2.27 ± 0.38 (6)
C	4.65 ± 0.78 (7)	7.2 ± 1.94 (7)	6.35 ± 1.46 (6)	3.23 ± 0.59 (6)
Gastro L	6.0 ± 2.2 (7)	3.3 ± 0.8 (7)	2.6 ± 0.5 (6)	1.4 ± 0.2 (6)
C	4.6 ± 2.4 (7)	4.2 ± 1.3 (7)	3.8 ± 0.9 (6)	1.2 ± 0.1 (6)

Number of measurements in each group in parentheses. L = muscles with common iliac artery ligated, C = contralateral muscles, TA = tibialis anterior, EDL = extensor digitorum longus, Gastro = gastrocnemius.
* Significantly different from contralateral muscles $P < 0.05$.

Table 3 Capillary supply in extensor digitorum longus (EDL) and subtypes of tibialis anterior (TA) muscles of the rat

	C/F ratio		
	EDL	*TA 'white'*	*TA 'red'*
Ligated + saline ($n = 7$)			
Control	2.01 ± 0.08	1.39 ± 0.08	2.47 ± 0.09
Ligated	2.04 ± 0.88	1.36 ± 0.08	2.48 ± 0.06
Ligated + torbafylline ($n = 7$)			
Control	2.11 ± 0.07	1.34 ± 0.03	2.40 ± 0.04
Ligated	2.07 ± 0.12	1.24 ± 0.03	2.44 ± 0.05
Ligated + pentoxyfylline ($n = 6$)			
Control	1.91 ± 0.08	1.24 ± 0.02*	2.43 ± 0.07
Ligated	2.05 ± 0.11	1.29 ± 0.05	2.46 ± 0.09
Ligated + buflomedil ($n = 6$)			
Control	1.95 ± 0.06	1.32 ± 0.04	2.37 ± 0.06
Ligated	1.95 ± 0.09	1.28 ± 0.03	2.36 ± 0.07

* $P < 0.05$ against control in ligated and saline.

ity in the development of collateral circulation: CV represented 60.4, 60.1, 117.8 and 51.5% in the control, torbafylline-, pentoxifylline- and buflomedil-treated animals, respectively.

Capillary supply

Capillary supply was estimated in TA and EDL as C/F ratio (Table 3). It was similar in all groups in control and ligated muscles, and no drug, with the exception of pentoxifylline, had any effect on C/F in either EDL or TA. There was a slightly but significantly lower C/F ratio in the glycolytic part of control tibialis in pentoxifylline-treated animals when compared to contralateral muscles in the control group.

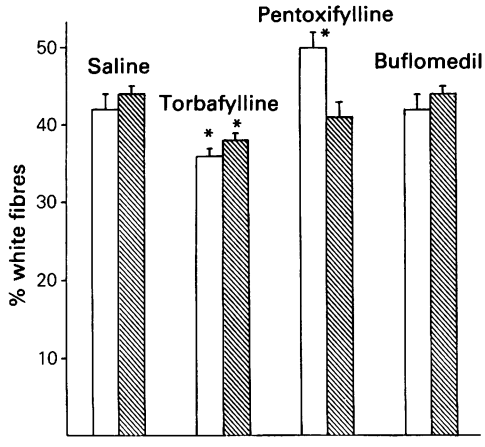

Figure 1 Proportion of white (glycolytic) fibres in rat extensor digitorum longus (EDL) with normal (control, open columns) and limited (ligated, hatched columns) blood supply. * Values significantly different from the control (saline) group.

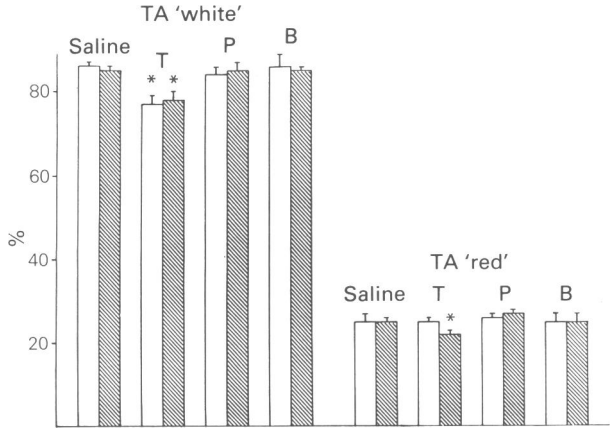

Figure 2 Proportion of glycolytic (white) fibres in the glycolytic cortex ('white') and oxidative core ('red') tibialis anterior (TA) muscles with normal (control, open columns) and limited (ligated, hatched columns) blood supply: T, torbafylline; P, pentoxifylline; B, buflomedil-treated rats. * Values significantly different from saline-treated (control) animals.

Proportion of glycolytic fibres

The proportion of glycolytic fibres was 42 ± 1% and 44 ± 1% in control and ligated EDL in the control group and in animals treated with buflomedil. Animals treated with torbafylline showed a significantly lower proportion of glycolytic fibres in both control and ligated muscles (36 ± 1 and 38 ± 1 respectively) while extensor digitorum longus in rats treated with pentoxifylline showed a higher proportion of glycolytic fibres (50 ± 2%) in control muscles and 41 ± 3% in the ligated ones (Figure 1). The proportion of glycolytic fibres in the tibialis anterior was higher in the predominantly glycolytic cortex (86 ± 1 and 85 ± 1), in the contralateral and ligated muscles in the control group with similar values in animals treated with pentoxifylline and buflomedil. It became significantly lower (77 ± 2% in contralateral and 78 ± 2% in ligated) in animals treated with torbafylline. This effect of torbafylline in the oxidative core of TA was seen in ligated muscles only (Figure 2) where the proportion of glycolytic fibres decreased from 25 to 22 ± 1%.

Discussion

The data in this paper show that circulation in rat skeletal muscles 5 weeks after the ligation of the common iliac artery was sufficient to maintain normal blood flow so that there was no loss of weight in gastrocnemius which is a muscle involved in the maintenance of posture. Fast contracting tibialis anterior and extensor digitorum longus showed a small degree of atrophy and a significantly lower flow. These findings are seemingly contradictory to the data of Nicholson *et al.* (1985) and Angersbach *et al.* (1988) who found lower blood flow in the rat gastrocnemius muscle 6–12 weeks after the ligation of the femoral artery. However, faster development of collateral circulation, judged by blood flow measurements during contractions, was described by Elander *et al.* (1985) 4 days after ligation of the common iliac artery in soleus (another postural muscle) than in EDL. Our previous findings (Hudlická & Torres, 1989) also showed a faster spontaneous development of collateral circulation in soleus. The discrepancies may be due to the methods used, ^{133}xenon clearance in Nicholson's experiments and microspheres in those by Elander and in this study. Administration of xenon into the muscle, particularly into a muscle as complex as gastrocnemius, may vary according to the fibre types, and it may be that if the isotope were placed in the glycolytic part of the muscle, flow would be found to be lower, in agreement with our results on EDL and TA.

Nevertheless, this would not explain the different results found in the present study on muscle weights. It is possible that the development of collateral circulation is different, particularly with respect to various muscles, depending on the site of ligation. However, this discrepancy can only be resolved by direct comparisons in the same strain of animals preferably in the same laboratory.

The administration of various drugs did not have any dramatic effect on either muscle atrophy or muscle blood flow. The weights of TA and EDL after ligation were not significantly different from the weights of contralateral muscles in animals treated with torbafylline (albeit the effect was very small) but were still smaller in animals treated with pentoxifylline or buflomedil. The difference between the resting blood flow in muscles with ligated common iliac arteries and contralateral muscles was no longer significant after drug treatment. This was due to a small, though insignificant, elevation of flow in the case of torbafylline and pentoxifylline, particularly in muscles with limited blood supply, but to a decrease in blood flow in contralateral muscles in the case of buflomedil.

Previous results have shown that torbafylline and pentoxifylline increase blood flow during acute infusion of $3\,mg\,kg^{-1}$ to a similar degree, both at rest and during muscle contractions in ischaemic muscles, but only torbafylline significantly diminished muscle fatigue. Torbafylline, but not pentoxifylline, also increased force of contraction in ischaemic muscles in a dose of up to $5\,mg\,kg^{-1}\,min^{-1}$ and increased surface Po_2 tension of ischaemic muscles. Further increase in dosage did not produce any greater changes, and it actually resulted in a decrease in Po_2 in animals treated with pentoxifylline, very probably due to decreased blood pressure. When these two drugs were given to rats for 16 days at the same dose as in the present experiments, torbafylline but not pentoxifylline, prolonged significantly the time the animals could run (Okyauz-Baklouti, 1988). It was therefore important to compare these doses of both drugs to find out whether the difference in improved performance could be explained either by better capillary supply or changes in fibre composition.

Capillary supply was not affected either by ligation or by drug treatment. Mäkitie (1977) described higher capillary/fibre ratio in gastrocnemius muscles in patients with intermittent claudication, but Henriksson *et al.* (1980) did not see any differences and Clyne *et al.* (1985) found lower values. It is difficult to find an explanation for such controversial results. Capillary supply is usually adapted to the level of oxidative metabolism, but it is still not quite clear whether this is decreased (e.g. Henriksson *et al.*, 1980) or increased (e.g. Bylund-Fellenius *et al.*, 1988) in muscles with limited blood supply. Moreover, increased capillarisation was found in muscles of animals treated with the xanthine derivative HWA 285, but the fibre composition was not changed and neither was muscle performance (Ziada *et al.*, 1984). The lack of increased capillarisation in muscles with intact blood supply after long-term administration of various drugs is in agreement with the lack of higher blood flow: increased capillarisation was usually found only in muscles of animals treated with drugs that produced a longlasting vasodilatation (Tornling *et al.*, 1980; Ziada *et al.*, 1984; Hudlická *et al.*, 1984). The fact that there was no capillary loss in muscles with limited blood supply would indicate that the collateral circulation and perfusion pressure were sufficient stimulus for the maintenance of the capillary structure.

The most pronounced difference in the effect of treatment with various drugs was observed on muscle fibre composition. Rat EDL is composed of approximately 40% glycolytic fibres (Ariano *et al.*, 1973), and this proportion was not changed by ligation. Neither was there a change in fibre composition in the predominantly glycolytic cortex or predominantly oxidative core of the tibialis anterior. These results are in agreement with the data on fibre type distribution in gastrocnemius muscles of patients with peripheral vascular disease (Henriksson *et al.*, 1980; Clyne *et al.*, 1982; Jansson *et al.*, 1988), but are at variance with findings reported by Hammarsten *et al.* (1980) who described a higher proportion of IIa (oxidative) fibres in the muscles of patients with intermittent claudication. Zeman *et al.* (1988) described a slight increase in the percentage of fast fibres and their hypertrophy in rat soleus and EDL after long-term treatment with a β_2-adrenoceptor agonist, clenbuterol. However, there are no data available on the effects of drugs on fibre composition in muscles with limited blood supply. In the present study, neither pentoxifylline nor buflomedil had any effect, while torbafylline significantly decreased the proportion of glycolytic fibres in all ligated muscles; it also decreased it in muscles or muscle parts with intact blood supply with a high proportion of glycolytic fibres. It may be argued that a higher dose of e.g. pentoxifylline could have had a similar effect to torbafylline. However, since the proportion of glycolytic fibres in normal muscles was significantly increased using the same dose of pentoxifylline as that of torbafylline, it is highly unlikely that any increase in dosage could reverse the effect of this drug.

Conversion of fibre types towards the oxidative form has so far only been described during endurance training (e.g. Saltin & Gollnick, 1983) and chronic electrical stimulation (e.g. Brown *et al.*, 1976). Increased oxidative capacity has also been achieved by administration of thyroid hormones (e.g. Winder *et al.*, 1980). Each of these conditions is very probably initiated by different signals and it would be premature to consider whether torbafylline may act by similar or different mechanisms.

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