Effects of ischaemia, blood loss and reperfusion on rat muscle protein synthesis, metabolite concentrations and polyribosome profiles *in vivo*

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In adult rat gastrocnemius muscles, on reperfusion after 45 min of tourniquet ischaemia, protein synthetic rates were depressed by over half for 1 h compared to normal (12 %/day), and were at least one-third below normal for up to 5 h afterwards. Ischaemia caused muscle concentrations of phosphocreatine to be depressed by 70 %, and those of lactate to be elevated by 350 %; the proportion of ribosomes as polyribosomes was decreased by half. Unlike the rates of protein synthesis, all of these variables returned to normal after 35 min of reperfusion. When 25 % of the blood volume was removed (for 10–45 min), there were falls in the rate of gastrocnemius protein synthesis and in phosphocreatine concentration, and an increase in lactate concentration. On blood replacement, protein synthesis and metabolite concentrations returned to normal within 15 min. Polyribosome profiles were unaffected by blood loss or replacement. There were highly significant correlations between the rate of gastrocnemius protein synthesis and both phosphocreatine concentration and 1/(lactate concentration), during blood loss and replacement, i.e. during both the fall and rise in muscle energy status. We conclude that the effects of ischaemia and blood loss on protein synthesis are not equivalent.

INTRODUCTION

Studies carried out using incubated (Ryan *et al.*, 1974) and perfused (Bylund-Fellenius *et al.*, 1981; Preedy *et al.*, 1984) preparations have suggested that changes in intramuscular energy status may affect the rate of muscle protein synthesis. Pathophysiologically, such changes might be brought about by reductions in tissue blood flow; reduced blood flow to skeletal muscle has been associated with the metabolic response to surgical trauma, which includes a depression of muscle protein synthesis (Lundholm *et al.*, 1982; Rennie *et al.*, 1984). Furthermore, muscle wasting is common in patients with peripheral vascular disease, with the degree of wasting said to be proportional to the severity of occlusion (Clyne, 1980), and we have preliminary evidence of a depression of muscle protein synthesis in such patients (MacLennan *et al.*, 1987).

In perfused muscle, low perfusate flow during electrically-stimulated contraction caused a fall in muscle phosphocreatine concentrations and protein synthesis rates without altering the muscle polyribosome density-gradient profile (Bylund-Fellenius *et al.*, 1981). These results suggested that protein synthesis was blocked by equal decreases in the rates of both initiation and elongation of polypeptide chains (Pain, 1978). This contrasts with the increases in the disaggregation of polysomes (indicating a failure of initiation) which occur in other circumstances of depression of muscle protein synthesis rate, such as lack of insulin *in vivo* (Harmon *et al.*, 1984) and *in vitro* (Jefferson, 1979).

It is tempting to relate the depression of protein synthesis observed in perfused muscle to the many

circumstances *in vivo* in which derangements of energy status are accompanied by muscle wasting (e.g. chronic bronchitis, emphysema, cardiac cachexia), but it seems premature to do so without more information. To obtain such information we designed experiments to discover whether, in rats *in vivo*, (1) skeletal muscle protein synthesis was depressed by alteration of energy status induced by ischaemia and reduced blood flow; (2) the changes were reversible; and (3) the effects of lack of oxygen differed in kind or simply in extent from those of inadequate delivery of oxygen.

The experiments described below were designed to investigate the effects of alterations in intramuscular energy status in rats *in vivo* on the protein synthesis rate (estimated by [³H]phenylalanine incorporation) in muscle. Perturbation of the muscle metabolic state was induced either by ischaemia with later reperfusion or by removal and replacement of blood. The effects of these treatments on the relative rates of initiation and elongation in gastrocnemius muscles were evaluated by construction of polyribosome profiles using density-gradient centrifugation. Indices of muscle metabolic state were obtained by measurement of muscle concentrations of phosphocreatine, ATP and lactate, and relationships were sought between these and protein synthesis rate.

EXPERIMENTAL

Materials

Radiochemicals were purchased from Amersham, Bucks., U.K. ([³H]phenylalanine) or from New England Nuclear, Boston, MA, U.S.A. (Sc⁴⁵-labelled micro-

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spheres, diameter $15.4 \pm 0.7 \mu\text{m}$). Sodium pentobarbital (Sagatal) was from May and Baker, Dagenham, Essex, U.K. All other chemicals and biochemicals were purchased from Sigma Chemical Co., Poole, Dorset, U.K. or BDH, Poole, Dorset, U.K. Female Wistar rats (170–210 g) (Bantin and Kingman Ltd., Hull, U.K.) were maintained on a 12 h light/dark cycle, with food and water available *ad libitum*.

Tissue sampling

The skin round the ankle joint of anaesthetized animals (pentobarbital, i.p., 4.8 mg/100 g of body weight) was cut free and the skin peeled back; a sample of superficial gastrocnemius was freeze-clamped, *in situ*, with aluminium block tongs previously kept in liquid N_2 .

Determination of protein synthetic rate

The determination of the rate of protein synthesis and its calculation were by methods described by McNurlan *et al.* (1982) and Garlick *et al.* (1980). Briefly, a solution of 150 mM- $[\text{^3H}]\text{Phe}$ (75–100 $\mu\text{Ci/ml}$) was injected intravenously (1 ml of solution/100 g of body weight). About 10 min later, tissues were sampled as described above; the exact time at which tissues were freeze-clamped was noted and subsequently used in calculations. Tissues were stored in liquid N_2 and then processed for determination of the specific radioactivity of free and protein-bound Phe.

It was considered desirable to measure muscle metabolite concentrations and protein synthesis rates in the same animals. Because accurate estimation of the intracellular concentrations, *in vivo*, of metabolites such as lactate and phosphocreatine (which undergo rapid changes in concentration *post mortem*) requires that tissues are freeze-clamped *in situ* (Lowry & Passonneau, 1972) it was necessary to measure the protein synthesis rate in anaesthetized animals. Preliminary experiments showed that Nembutal anaesthesia for periods of up to 1 h did not alter the protein synthetic rate in gastrocnemius muscles of fed rats [unanaesthetized: 10.6 ± 3.2 ($n = 4$); anaesthetized: 10.7 ± 2.0 ($n = 4$); means $\pm$ S.D.]

Polyribosome profiles

Preparation of polyribosome profiles from freeze-clamped gastrocnemius muscles was as described by Harmon *et al.* (1984), except that 10 U/ml of RNAase inhibitor (RNAase Inhibitor 1, Sigma Ltd.) was included during homogenization. After centrifugation, the gradient was drawn from the bottom of the centrifuge tube by suction, fractions were collected and absorbance at 260 nm was measured. The position of the 80 S ribosomal peak was verified by preparing profiles of polyribosomes from gastrocnemius muscle from 24 h-fasted rats. In such preparations the 80 S peak is especially prominent (Harmon *et al.*, 1984). The relative areas of the constituents of the profile in the experimental preparation were evaluated by gravimetric integration of the areas beneath the absorbance curve.

The percentage of ribosomes in the form of polyribosomes was defined as: $[\text{polyribosomal area} / (\text{polyribosomal area} + 80 \text{ S monomer area})] \times 100 \%$.

Cardiac output and regional blood flow

Cardiac output and regional blood flow were measured with radioactive microspheres by the method described

by Rothwell & Stock (1981). Reference samples were withdrawn over 1 min from the femoral artery.

Metabolites in muscle

Muscle metabolite concentrations were determined on perchloric acid-deproteinized, neutralized extracts (Lowry & Passonneau, 1972) of freeze-clamped muscle. Lactate was determined according to Gutman & Wahlefeld (1974). The spectrophotometric methods for determination of phosphocreatine, ATP and creatine, and the fluorimetric method for determination of pyruvate, are described by Lowry & Passonneau (1972). Protein was determined by the method of Lowry *et al.* (1951).

Ischaemia and reperfusion

Ischaemia was induced by tying a length of twine tightly around the left thigh of anaesthetized rats. After 45 min, the tourniquet was removed and protein synthesis rates, metabolite concentrations and polyribosome profiles were determined on muscle samples taken from groups of animals at intervals of up to 48 h after tourniquet removal.

Blood loss and replacement

The left carotid artery of each anaesthetized animal was cannulated and an extension filled with heparinized 150 mM-NaCl was fitted. Then, 25 % of an animal's blood volume was removed (20 ml/kg of body weight) via the carotid cannula after discarding the dead-space fluid. Heparinized 150 mM-NaCl was injected into the extension set after blood removal to maintain patency of the cannula. In some experiments the blood was withdrawn into a heparinized syringe, maintained at 37 °C for 30 min, then reinjected via the carotid cannula after discarding the dead-space fluid.

Muscle protein synthesis rates, metabolite concentrations, gastrocnemius polyribosome profiles and cardiac output and muscle blood flow were measured at intervals over 45 min after blood removal and also 15 min after the replacement of blood.

Statistical analysis

Statistical analysis was by Student's unpaired *t* test.

RESULTS

Effects of ischaemia and reperfusion

Ischaemia for 45 min caused a depression of phosphocreatine concentration (from 76 ± 19 to $33 \pm 6 \mu\text{mol/g}$ of protein; means $\pm$ S.D., $P < 0.001$) and an elevation of lactate (from 9.6 ± 1.8 to $29.8 \pm 10 \mu\text{mol/g}$ of protein; means $\pm$ S.D., $P < 0.001$) in the gastrocnemius muscle of the ischaemic limb. Concentrations of these metabolites in muscles of the contralateral non-ischaemic limb were unaffected. Muscle ATP concentrations were unaffected ($31.0 \pm 5.2 \mu\text{mol/g}$ protein; mean $\pm$ S.D.). During reperfusion after ischaemia, phosphocreatine and lactate concentrations in gastrocnemius muscles of the previously ischaemic limb returned to normal within 40 min (Fig. 1). Protein synthesis rates in gastrocnemius muscles from the previously ischaemic limb were depressed during the first hour of reperfusion but returned to normal after reperfusion for between 1 and 5 h (Fig. 1). Protein synthesis rates and metabolite concentrations in

Fig. 1. Effects of ischaemia and reperfusion on gastrocnemius protein synthesis rate and metabolite concentrations.

For details of the application and removal of tourniquet ischaemia, see the Experimental section. Results from control (●) and ischaemic (▲) limbs are shown. All values are means $\pm$ S.D.; $n = 4-6$. * ($P < 0.05$) and ** ($P < 0.001$) indicate statistically significant differences from control values.

gastrocnemius muscles of the non-ischaemic limbs were unaffected.

In gastrocnemius muscles, 10 min after tourniquet removal, a depression of protein synthesis rate was accompanied by a decrease in the proportion of ribosomes in the form of polyribosomes (Figs. 2 and 3). By 40 and 60 min after tourniquet removal, polyribosome profiles had returned to normal, although protein synthesis rate remained depressed (Fig. 1).

Under no circumstances were muscle concentrations of total creatine (i.e. free-plus-phosphocreatine) altered.

Effects of blood loss and replacement

Removal of 25 % of total blood volume brought about depressions of cardiac output and blood flow to hindlimb skeletal muscle between 10 and 45 min after removal. Following blood replacement, hind-limb blood flow returned to normal within 15 min (Fig. 4).

After blood loss, the concentration of phosphocreatine fell and that of lactate rose in gastrocnemius muscles

Fig. 2. Ribosome profiles from rat gastrocnemius muscle

Muscle was sampled from rats studied without intervention (control) or after 10 or 40 min of blood reperfusion after ischaemia.

Fig. 3. Effects of ischaemia and reperfusion on gastrocnemius muscle polyribosome profile

For details of application and removal of tourniquet ischaemia, see the Experimental section. Results from control (□) and post-ischaemic (■) muscle are shown. All values are means $\pm$ S.D., $n = 3-4$. ** ($P < 0.001$) indicates that the proportion of ribosomes as polyribosomes after 10 min reperfusion was significantly lower than that in controls.

Fig. 4. Effects of blood removal and replacement on skeletal muscle blood flow and cardiac output

For details of blood manipulation, see Experimental section. All values are means $\pm$ S.D., $n = 4-5$. * ($P < 0.05$) and ** ($P < 0.001$) indicate significant differences between controls and muscles of the same type taken from animals from which blood had been removed.

(Fig. 5). When blood was replaced, the concentrations of lactate and phosphocreatine in gastrocnemius returned to normal values within 15 min. The muscle total creatine concentration was unaltered by ischaemia and reperfusion (results not shown).

Protein synthetic rates were depressed in gastrocnemius (Fig. 5) after blood loss. When blood was replaced, protein synthesis rates returned to normal within 15 min. Despite the fall in protein synthesis rate after blood loss, there was no change in the proportion of gastrocnemius ribosomes in the form of polyribosomes (Fig. 5).

There were significant correlations (Fig. 6) between protein synthesis rates and phosphocreatine concentration and between protein synthesis rates and the reciprocal of lactate concentration in gastrocnemius muscles after blood loss and replacement. The relationship with protein synthesis was the same during the fall and the rise of phosphocreatine and lactate concentrations.

DISCUSSION

Both blood loss and total ischaemia brought about changes in energy status and protein synthesis rate in gastrocnemius muscle. However, although the depressions in protein synthesis rate and in muscle phosphocreatine concentration were of similar magnitude in both situations, blood replacement resulted in normalization of the rate of protein synthesis within 15 min, whereas during reperfusion after total ischaemia, protein synthesis remained depressed for 1-5 h.

Because the creatine kinase reaction remains at equilibrium during a variety of metabolic disturbances involving a fall in phosphocreatine in skeletal muscle (e.g. Shoubridge *et al.*, 1985) and since we found the total

Fig. 5. Effects of removal of blood and its replacement on indices of protein synthetic activity and intermediary metabolism in gastrocnemius muscle

See Experimental section for details. The broken lines and the open bar show changes after blood replacement. All values are means $\pm$ S.D.; $n = 4-5$ except for the polyribosome value for the blood replacement group which is the mean of $n = 2$. The bars (hatched or open) show mean rate of protein synthesis over the periods shown. * ($P < 0.05$), ** ($P < 0.01$) indicate statistically significant differences from time zero values.

creatine concentration to be constant, it is likely that the muscle phosphocreatine concentration was directly proportional to the muscle ATP/ADP ratio and therefore an indication of the phosphorylation potential or energy status of the muscle. However, we recognize that the relationship between the ATP/ADP ratio and the phosphocreatine/creatine ratio might have been altered by other factors (such as changes in pH or free Mg^{2+} concentration) which were not evaluated in our experiments.

The fact that phosphocreatine concentration and protein synthesis rate both returned to normal within 15 min of blood replacement suggests that the protein synthetic rate was affected by muscle energy status under these circumstances. Gastrocnemius polyribosome profiles remained unaffected by blood loss and replacement, despite a decrease in protein synthesis rate. This decrease appears to have been brought about by falls of equal

Fig. 6. Relationships between (a) phosphocreatine concentration and protein synthesis rate and (b) lactate concentration and protein synthesis rate in left and right gastrocnemius muscles respectively

The relationships are shown for controls (○); 10–45 min after removal of 25% blood volume (●) and 15 min after replacement of 25% of blood volume following its removal of 30 min (△). Superficial gastrocnemius protein synthesis rate (%/day) = $0.12 \times [\text{phosphocreatine concentration } (\mu\text{mol/g protein})] + 2.62$; $r = 0.67$, $P < 0.001$. Also, the log of the superficial gastrocnemius protein synthesis rate (%/day) = $0.25 \times [\text{lactate concentration } (\mu\text{mol/g protein})] + 13.1$; $r = -0.62$, $P < 0.001$.

magnitude in the rates of initiation and elongation of polypeptide chains. There is evidence that both processes may be controlled by energy status *in vitro* (Tarrago *et al.*, 1973; Walton & Gill, 1976).

Although relationships between muscle protein synthesis rate and phosphocreatine or lactate concentrations have previously been demonstrated during a fall in muscle energy status (Bylund-Fellenius *et al.*, 1981), the present results are the first of which we are aware showing that, on restriction of oxygen supply, protein synthesis rates and intramuscular phosphocreatine and lactate concentrations may return to their control values without hysteresis. This is stronger evidence for a causal relationship between energy status and the rate of protein

synthesis than if a correlation were displayed only when energy status was altered in a single direction.

The effects of ischaemia were different from those of blood loss and replacement. During reperfusion after total ischaemia, the superficial gastrocnemius phosphocreatine concentration returned to normal before muscle protein synthesis rate, suggesting that inhibition of synthesis was not immediately relieved by the return of the muscle energy status to normal. Furthermore, the fall in synthesis rate was accompanied by a decrease in the proportion of ribosomes in the form of polyribosomes, although this returned to normal before the protein synthesis rate fully recovered. It appears, therefore, that on tourniquet removal decreased protein synthesis was the result of a greater fall in the rate of initiation than of elongation. As a consequence, it is reasonable to suggest that the block in protein synthesis arising from complete ischaemia included mechanisms additional to those through which protein synthesis was blocked after partial blood loss.

The lack of insulin availability during ischaemia might have been important: it is recognized that removal of insulin produces a fall in protein synthesis arising from a greater fall in the rate of initiation than of elongation (see Introduction).

These results imply that during decreases in protein synthesis induced by changes in energy status alone, the ribosomal aggregation state is unaltered: thus the block in synthesis during low blood flow appears to be due to equal decreases in the rates of initiation and elongation of polypeptide chains. Previous work has left unresolved the question of ribosomal aggregation state during depressions of protein synthesis associated with changes to energy status (Oler *et al.*, 1969; Jefferson *et al.*, 1971; Freudenberg & Mager, 1971; Van Vennoij *et al.*, 1972; Ayuso-Parrilla & Parrilla, 1975; Bylund-Fellenius *et al.*, 1984).

The question therefore remains as to whether the decreased protein synthesis rate is simply limited by the availability of ATP during conditions of altered energy status in muscle, is due to a decrease in the ATP/ADP ratio or a fall in the free energy associated with ATP hydrolysis, or is the result of a change in some other factor, induced by alterations in oxygen delivery, which inevitably reduces protein synthesis.

Changes in muscle energy status often accompany circumstances in which amino acid release from muscle to the circulation would be beneficial to the organism as a whole. For instance, after blood loss, amino acids released from muscle may be used for hepatic synthesis of acute phase proteins or as a fuel source for other tissues. There is substantial evidence that when changes in muscle mass take place physiologically, protein synthesis is the facilitative process (i.e. it increases during growth and decreases during wasting) with protein breakdown being adaptive to these changes (reviewed by Millward *et al.*, 1983). It seems likely that acute modulation of amino acid release from muscle as a result of poor oxygen delivery would also be affected primarily by depressions in protein synthetic rate, rather than by increases in protein breakdown, especially as proteolysis is ATP-dependent (Goldberg & St John, 1976). It is, therefore, conceivable that changes in muscle energy status may act as a signal through which amino acids are made available from muscle protein via decreases in muscle protein synthesis.

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