Ischaemic preconditioning of skeletal muscle

1. PROTECTION AGAINST THE STRUCTURAL CHANGES INDUCED BY ISCHAEMIA/REPERFUSION INJURY

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Ischaemic preconditioning is a process by which exposure of a tissue to a short period of non-damaging ischaemic stress leads to resistance to the deleterious effects of a subsequent prolonged ischaemic stress. It has been extensively described in the heart, but few studies have examined the possibility that it can occur in skeletal muscle. We have used a rat model of ischaemia of one limb to examine this possibility. Exposure of the hind limb to a period of ischaemia of five minutes and reperfusion for five minutes significantly protected the tibialis anterior muscle against the structural damage induced by a subsequent period of limb ischaemia for four hours and reperfusion for one hour. This protection was evident on examination of the muscle by both light and electron microscopy. Longer or shorter times of prior ischaemia had no effect.

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Skeletal muscle is known to be relatively resistant to ischaemic injury in comparison with other tissues, but injury may occur after prolonged use of a pneumatic tourniquet or after arterial occlusion.^{1,2} Most of the tissue injury which is apparent after ischaemia is sustained at the time of reperfusion and the mechanisms underlying this process have been the subject of considerable study.^{3,4} Procedures which reduce the effects of ischaemia in the limbs will be valuable in clinical practice and allow safer use of the tourniquet. In skeletal muscle, previous data indicate that oxygen radicals

produced during reperfusion may play a role in the damage.⁵⁻⁷ The source of these radicals is unclear, although by analogy with other tissues, both generation of superoxide radicals by xanthine oxidase activity⁸ and generation of radicals by neutrophils or macrophages sequestered within the reperfused tissue⁹ may be important. Bulkley⁴ has suggested that these putative mechanisms are linked in such a way that superoxide produced from xanthine oxidase in the reperfused vascular endothelial cell causes upregulation of adhesion molecules on the luminal surface of the endothelial cell. These molecules react with complementary ligands on circulating neutrophils, which are consequently arrested and activated releasing damaging proteases and oxidants.

Our previous data which examined a rabbit model of ischaemia and reperfusion damage to limb skeletal muscle, are in accord with this hypothesis since both antioxidants and corticosteroids were found to reduce the structural damage which occurred after reperfusion.¹⁰

Recent studies on cardiac and other tissues have indicated that short periods of non-damaging ischaemic stress produce an adaptive response in the heart which results in resistance to the normally damaging effects of a subsequent prolonged ischaemic stress.¹¹ This phenomenon is known as 'ischaemic preconditioning'. In cardiac tissue protection by ischaemic preconditioning can be obtained after a single period of ischaemia for five minutes. The reflow period after this brief ischaemia may be as short as one minute, but protection is reduced after reperfusion for one hour.¹¹ Very few studies of this type have been undertaken in skeletal muscle, but one report in the pig indicated that three cycles of ischaemia for ten minutes and reperfusion for ten minutes could protect skeletal muscle against subsequent ischaemic damage.¹²

Our aim therefore was to determine whether a single short ischaemic period could precondition rat skeletal muscle against the structural damage normally induced by a prolonged period of ischaemia for four hours and reperfusion for one hour, and to use this rodent model to investigate the potential mechanisms involved (see part 2 pp 1189).

Materials and Methods

We used 48 male Wistar rats weighing approximately 300 g which were maintained on a standard laboratory diet. All

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animals were fully anaesthetised with fentanyl-fluanisone (0.3ml/kg, Hypnorm) and diazepam (2.5 mg/kg, Valium) throughout the experiment. Anaesthesia was maintained by supplemental fentanyl-fluanisone (0.3 ml/kg) every 30 minutes. The animals underwent a period of ischaemia and reperfusion on one limb as previously described.¹³ A small incision was made in the skin and the femoral artery and vein identified. Ischaemia of the limb was induced by clamping the femoral artery. Heating coils were placed around the ischaemic and contralateral limbs to maintain a skin temperature of 35°C. Six animals were anaesthetised, but not subjected to ischaemia of the limb, acting as a non-ischaemic control group.

Anaesthesia was maintained until the end of the experiment when the animals were killed by an overdose of anaesthetic and the tibialis anterior and extensor digitorum longus (EDL) muscles were removed. Strips of the tibialis anterior muscle 5 mm long were fixed in 3% glutaraldehyde for microscopic analyses and the remainder was mounted on a cork block and frozen in isopentane cooled in liquid nitrogen. This latter sample was used for immuno-cytochemical analyses and for measurement of the composition and size of muscle fibres. The EDL muscles were placed rapidly in bicarbonate-buffered mammalian Ringer's solution and maximum *in vitro* force production measured.

Microscopic analysis. The fixed strips of tibialis anterior muscle were post-fixed in buffered osmium tetroxide, dehydrated in alcohols of increasing strength and embedded in Epon Araldite (Agar Scientific Ltd, Stansted, UK). Semi-thin longitudinal sections of 1.5 to 2.0 mm were stained using 1% Toluidine Blue (Gurr, BDH Ltd) in 1% Borax buffer. The extent of damage to fibres was quantified from the semi-thin section by point counting using a square lattice grid as previously described.¹⁰ Six sections from each muscle were examined, except for two in which for technical reasons only five were examined. Thin sections of 90 to 120 nm were cut for transmission electron microscopy and mounted on 200 mesh copper grids. Grids were stored with uranyl acetate and lead citrate and examined in a Phillips CM-10 electron microscope (FEI, Electron Optics, Eindhoven, The Netherlands).

Measurements of muscle force production. The isolated EDL muscles were mounted between platinum plate electrodes on a glass holder and placed in bicarbonate-buffered mammalian Ringer's solution, oxygenated with 95% O₂ and 5% CO₂ and maintained at 37°C. One tendon was attached to a force transducer. The optimum length for force production was adjusted while monitoring the maximum twitch force and the maximum tetanic force was obtained by stimulation with square-wave pulses of 0.1 msec at 100 Hz and 70 volts for 0.5 seconds.

Preconditioning of the limb. Groups of six animals had preliminary periods of ischaemia induced in the limb before the main damaging period by clamping of the femoral artery. The different periods of preliminary ischaemia and reperfusion which were examined are shown in Table I.

Table I. Lengths of preliminary periods of ischaemia and reperfusion imposed before the main ischaemic episode of four hours

Ischaemic time (min)	Reperfusion time (min)
2	5
3	5
5	5
5	15
7	5
10	5

Table II. Effect of varying the preliminary periods of ischaemia and reperfusion on the proportion of fibres in the anterior tibialis damaged after four hours of subsequent limb ischaemia and one hour of reperfusion. Data are presented as the median (75 percentile range), with six animals at each time point

Duration of preliminary ischaemia/reperfusion (min)	Reperfused limb (% damaged sites)
0/0	59.1 (51.9 to 70.7)
2/5	48.1 (30.5 to 59.4)
3/5	66.2 (64.9 to 67.7)
5/5	31.1 (29.1 to 31.7)*
5/15	69.0 (40.6 to 70.8)
7/5	64.8 (42.1 to 83.3)
10/5p	61.2 (44.4 to 59.3)

*values significantly different from muscles not subjected to the preliminary period of ischaemia/reperfusion (0/0), $p = 0.0049$

Statistical analysis. The data are presented as the median (75 percentile range). All results were analysed by the Kruskal-Wallis test for non-parametric data followed by *post-hoc* comparisons with Bonferroni correction between groups which appeared to be significant in the initial analysis.

Results

Four hours of limb ischaemia followed by one hour of reperfusion caused similar changes to the tibialis anterior muscle compared with those previously reported in a rabbit model.^{10,14} These included mitochondrial swelling, a dilated sarcoplasmic reticulum and disruption of the myofibrillar structure on electron-microscopic examination and clear loss of structural integrity, areas of hypercontraction and disruption of the striations on light microscopy (Figs 1 and 2). Point counting of semi-thin sections revealed damaged sites in the reperfused muscles of 59.1% (51.9 to 70.7).

Effect of a preliminary ischaemic period. The effect of various times of preliminary ischaemia and reperfusion on the extent of damage to the tibialis anterior muscle is shown in Table II. A significant reduction in the amount of damaged sites was seen in muscles which had been subjected to five minutes of ischaemia followed by five minutes of reper-

Fig. 1a

Fig. 1b

Fig. 1c

Photomicrographs of typical semithin sections of rat anterior tibialis muscles showing a) control muscle, b) non-preconditioned reperfused muscle, and c) reperfused muscle subjected to a preconditioning protocol of ischaemia for five minutes and reperfusion for five minutes (Toluidine Blue; x 140).

fusion. Shorter or longer periods of ischaemia did not provide significant protection and when the reperfusion period was increased from 5 to 15 minutes the protection offered by five minutes of preischaemia was lost. Typical semi-thin sections from control animals and those subjected to five minutes of preischaemia and five minutes of reperfusion are shown in Figure 1. The typical electron-microscopic appearance of these muscles is shown in Figure 2. Pre-ischaemia for five minutes also conferred protection against the ultrastructural damage visible at this level.

Control EDL muscles produced a mean maximum twitch force of 26.0 ± 2.3 mN (SEM) and a mean tetanic force of 337 ± 51 mN. Most reperfused muscles could not be stimulated to produce force even when the applied voltage was increased and this was unaffected by any of the pre-ischaemia protocols. Occasional reperfused muscles produced very small amounts of force (<5% of control), but this was in less than 5% of the muscles studied and there was no consistent pattern concerning those muscles (i.e. preconditioned or control) which could be activated.

Discussion

Our data have indicated that the tibialis anterior muscle of the rat hind limb can be preconditioned by a single short period of ischaemia of five minutes and reperfusion for five minutes against the damaging effect of a subsequent four-hour period of ischaemia and reperfusion for one hour (Table II, Figs 1 and 2). This is in general agreement with recently published work^{12,15} although in those studies mul-

tiple (four) short periods of ischaemia and reperfusion were used as the preconditioning stress.

A substantial number of studies of cardiac muscle have shown that short periods of ischaemia can precondition that tissue against reperfusion damage. We had hypothesised that the relative resistance of skeletal muscle to ischaemic injury indicated that longer periods of ischaemia would be necessary to precondition skeletal muscle. However, longer periods of ischaemia (7 to 10 minutes) were found to have no effect, but ischaemia of a similar duration to that previously found to reduce damage in cardiac muscle¹¹ was also effective in our skeletal muscle model.

Although the structural damage to the muscle was reduced by preconditioning, these procedures had no effect on the complete loss of force generation produced by the four hours of ischaemia. The loss of force generation by reperfused muscles has been reported previously^{16,17} and attributed to neuromuscular failure.¹⁶ Our studies of muscle force generation in vitro suggest that an inability to excite the muscle membrane may be responsible and may be analogous to the process of 'stunning' or post-ischaemic dysfunction which occurs in cardiac tissue.¹⁸ In that case, the dysfunction would be expected to be reversible over a relatively short period of time, although we have not had the opportunity to study this possibility.

The mechanisms by which preconditioning protects skeletal muscle are addressed in part 2 (pp 1189) but there are general indications concerning mechanisms which can be obtained from the data presented here. Clearly, the ischaemic stress which provides the protection is relatively short

Fig. 2a

Fig. 2b

Fig. 2c

Electron micrographs of the typical ultrastructural appearance of rat anterior tibialis muscles showing a) control muscle, b) non-preconditioned reperused muscle, and c) reperused muscle subjected to a preconditioning protocol of ischaemia for five minutes and reperfusion for five minutes (x 1000).

and of equivalent time to that observed in cardiac muscle. This implies that it must be unrelated to the rate of loss of energy supply to the muscle during ischaemia since this loss in contracting cardiac muscle is considerably faster than that from skeletal muscle at rest. In addition, the protective effect is lost if the reperfusion period is extended to 15 min-

utes before induction of the main damaging ischaemia. Thus, a transient stress is required and the effect can be reversed by prolonged reperfusion. This argues against the protection observed being due to the preconditioning ischaemia stimulating expression of any transcribed protective factor (e.g. heat shock proteins) within the tissue. One possibility which has been extensively examined for cardiac tissue is that the ischaemic preconditioning causes release of adenosine which mediates the protection observed.¹¹ Our data are in accord with a similar mechanism occurring in skeletal muscle.

In conclusion, we have demonstrated that rat skeletal muscle can be protected against the damage induced by prolonged ischaemia and reperfusion by preconditioning with a short period of ischaemia of five minutes and a short period of reperfusion of five minutes. This has potential application in clinical procedures in man in which it may be possible to protect the limb during prolonged application of the pneumatic tourniquet. However, carefully controlled trials will be necessary to ensure that the effect in man is similar to that seen in the rat. If it is, routine preconditioning may be used for all operations in which the tourniquet time exceeds one hour. It may well reduce postoperative swelling and muscle pain. The use of a tourniquet, particularly for total knee replacement, is being questioned and this type of prophylaxis may sway the balance back towards its use for this procedure.^{19,20}

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