Ischaemic preconditioning of skeletal muscle

2. INVESTIGATION OF THE POTENTIAL MECHANISMS INVOLVED

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We have previously shown that prior exposure of rat hind limbs to ischaemia for five minutes and reperfusion for five minutes reduced the structural damage to skeletal muscle which followed a subsequent period of ischaemia for four hours and reperfusion for one hour. We have now examined the potential mechanisms by which this ischaemic preconditioning protocol may be effective in reducing damage to skeletal muscle induced by prolonged ischaemia and reperfusion. Prior exposure of the hindlimb to ischaemia for five minutes and reperfusion for five minutes did not prevent the fall in the ATP content of tibialis anterior which occurred after a subsequent period of ischaemia for four hours and reperfusion for one hour. Similarly, no effect of the preconditioning protocol was seen on the elevated muscle myeloperoxidase, indicative of an elevated neutrophil content, or abnormal muscle cation content. Reperfused ischaemic muscle was also found to have an increased content of heat-shock protein (HSP) 72, but the preconditioning protocol did not further increase the content of this or other HSPs indicating that it was not acting by increasing the expression of these cytoprotective proteins. The protective effects of preconditioning appeared to be mimicked by the infusion of adenosine to animals immediately before exposure to the four-hour period, indicating a potential mechanism by which skeletal muscle may be preconditioned to maintain structural viability.

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Ischaemic preconditioning can occur in many tissues, and studies reported in the accompanying paper (part 1, pp 1184) have shown the protective effect of a short period of ischaemia for five minutes and reperfusion for five minutes on rat skeletal muscle subjected to a subsequent period of damaging ischaemia for four hours and reperfusion for one hour. The biochemical mechanisms underlying the damage to skeletal muscle which follows ischaemia and reperfusion appear to involve a primary loss of supplies of muscle energy followed by a generation of oxygen radicals with a resultant endothelial dysfunction and neutrophil sequestration with release of further oxygen radicals, elastase etc.1-3

Data from other tissues have indicated that there are two main possible mechanisms by which preconditioning may inhibit subsequent damage during reperfusion. It has been proposed that the preconditioned tissue produces substances which help to protect it against injury. The most frequently considered protective substance has been adenosine, but bradykinin, nitric oxide and certain prostanoids have been suggested as mediators.4 Adenosine, in particular, is an attractive possibility for such a role since it is released by ischaemic tissue and when infused into ischaemic tissue prevents leukocyte sequestration,5 increases the activity of some antioxidant enzymes6 and preserves ATP levels.7

Alternatively, cells respond to all stresses by induction of ‘heat-shock’ or stress proteins and one or more of these proteins appears to play a role in protecting the cell against subsequent stress. Hypoxia has been shown to induce the synthesis of heat-shock proteins (HSPs) in cardiac tissue and this has been proposed as a potential mechanism for the protective effect of preconditioning.8

Our aim was to examine the mechanisms by which the ischaemic preconditioning of skeletal muscle described in the accompanying paper (part 1, pp 1184) prevents damage to skeletal muscle induced by four hours of ischaemia and subsequent reperfusion.

Materials and Methods

We used 22 male Wistar rats. One hind limb of the anaesthetised animals was subjected to periods of ischaemia and reperfusion by clamping of the femoral artery and vein as described in the accompanying paper (part 1, pp 1184). At the end of the experiment samples of the tibialis anterior...
muscle were prepared for microscopic examination as before and the remainder of the muscle was frozen rapidly in liquid nitrogen and stored at –70°C for biochemical analysis.

**Adenosine infusion.** In order to evaluate the potential of adenosine to mimic preconditioning a bolus injection of adenosine in isotonic normal saline at a dose of 1 mg/kg body-weight was given into the carotid artery of four rats one minute before clamping the femoral artery and vein.

**Biochemical analyses.** The ATP content of a perchloric acid extract of the muscle samples was analysed by an enzymic method. The myeloperoxidase activity was analysed as an index of the extent of neutrophil sequestration by the method described by Seekamp et al. Muscle cation (Ca, Mg, Na, K) content was analysed on freeze-dried samples as described by Jackson, Jones and Edwards.

For analysis of HSPs, muscle samples were homogenised in a range of protease inhibitors and proteins and analysed for HSP60, HSP72 (the inducible form of HSP70) and HSC70 (the constitutive form of HSP70) by SDS-PAGE followed by western blotting techniques using monoclonal antibodies obtained from Bioquote Ltd (York, UK), Sigma Chemical Co (Poole, UK), and Amersham Life Sciences (Amersham, UK). The intensity of staining for individual HSPs was quantified by densitometry.

All reagents used for these analyses were of Analar grade or the highest grade commercially available.

**Statistical analysis.** 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

**Effect of preconditioning on biochemical changes in reperfused tissue.** The ATP content of tibialis anterior muscles subjected to four hours of ischaemia and subsequent reperfusion with or without the protective preconditioning protocol (prior exposure to ischaemia for five minutes and reperfusion for five minutes) are shown in Table I. Muscles subjected to ischaemia for four hours and reperfusion for one hour lost approximately 30% of their total ATP content and this was not reversed by the preconditioning protocol.

The myeloperoxidase activity of muscles is shown in Table II. The activity of the enzyme was significantly increased in the reperfused limb of non-preconditioned animals. This was not significantly reduced in the animals given ischaemic preconditioning for five minutes. The injury to the rat hind limb muscles which followed reperfusion was also associated with an increase in muscle calcium and sodium and a decrease in muscle potassium as previously reported. The preconditioning protocols had no significant effect on these changes.

**Effects of adenosine infusion.** Adenosine was infused into the carotid artery immediately before clamping the femoral artery. It appeared to protect the tibialis anterior muscle significantly against reperfusion injury as assessed on semi-thin sections and electron micrographs (Figs 1 and 2). Quantitatively, the semithin sections of tibialis anterior muscle showed 3.2% of damaged sites (1.6 to 4.0) compared with 59.1% (51.9 to 70.7) in the control reperfused muscle. Treatment with adenosine also prevented the fall in ATP content (Table I) and the elevation of muscle myeloperoxidase.

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<tr>
<th>Table I. The ATP content of tibialis anterior muscles from animals subjected to ischaemia and reperfusion of one hind limb. Data are presented as median (75 percentile range)</th>
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<tr>
<td><strong>Treatment</strong></td>
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<td>None (n = 6)</td>
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<tr>
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<tr>
<td>5 min ischaemia/5 min reperfusion (n = 6)</td>
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<tr>
<td>Adenosine-treated (n = 4)</td>
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<td>*value significantly (p = 0.0066) different from control contralateral limb</td>
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<td>†value significantly (p = 0.0022) different from reperfused non-preconditioned limb</td>
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<th>Table II. Myeloperoxidase activity (ΔA/min/g. muscle) of tibialis anterior muscle from animals subjected to ischaemia and reperfusion of one hind limb. Data are presented as median (75 percentile range)</th>
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<td>*value significantly (p = 0.0004) different from control contralateral limb</td>
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<tr>
<td>†value significantly (p = 0.0069) different from control contralateral limb</td>
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<td>‡value significantly (p = 0.0246) different from reperfused non-preconditioned limb</td>
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dase activity associated with reperfusion injury (Table II). **Effect of preconditioning on muscle HSP content.** The effect of reperfusion injury and the preconditioning protocols on HSP60, HSP70 and HSC70 in tibialis anterior muscle is shown in Figures 3 and 4. Ischaemia for four hours and reperfusion for one hour alone resulted in a significant increase in the muscle content of HSP70. The preconditioning protocol did not induce any further increase in muscle HSP content, but conversely prevented the significant rise of HSP70 suggesting that these changes were induced by the damage which occurred on reperfusion of the non-preconditioned muscle.

**Discussion**

Our data indicate that the fall in muscle ATP content caused by ischaemia for four hours and reperfusion for one hour in the rat was not prevented by the preconditioning protocol which reduced the structural damage to the tissue (part 1, pp 1184). Similarly, the effective preconditioning protocol had no significant effect on the myeloperoxidase content of the muscle. No evidence for an induction of HSPs by the successful preconditioning protocols was obtained, but infusions of adenosine appeared to mimic the protective effect of preconditioning against reperfusion injury.

The infusion of adenosine has been shown to have a protective effect against reperfusion injury to cardiac tissue and it is postulated that adenosine is released from ischaemic cardiomyocytes and mediates the preconditioning effect. It is tempting to speculate that our data indicate that adenosine plays a similar role in the ischaemic preconditioning of skeletal muscle, but it is not immediately apparent whether adenosine will be released from skeletal muscle during short-term ischaemia (five minutes) and hence available for such a role.

The breakdown of ATP occurs during ischaemia with the production of adenosine monophosphate [AMP] by all tissues. In skeletal muscle the major route of breakdown of AMP is via adenylyl deaminase to inosine monophosphate with eventual formation of inosine and ultimately hypoxan-
thine. An alternative pathway involves the dephosphorylation of AMP to adenosine with subsequent conversion by adenosine deaminase to inosine etc. The extent to which this latter pathway occurs in skeletal muscle is unclear. Ronquist et al. have reported that the adenosine content of human smooth muscle was 70-fold higher than that of striated muscle and Fishbein, Davis and Foellmer have shown that AMP deaminase was present at 15- to 500-fold greater activity in skeletal muscle then in cardiac muscle. These data therefore indicate a relatively low potential of skeletal muscle to produce adenosine during ischaemia. By contrast, Hellsten and Frandsen have proposed that skeletal muscle cells in culture have ectoenzymes involved in the conversion of ATP, ADP and AMP to adenosine on the outside of the cells.

Another factor which argues against the release of adenosine from skeletal muscle as a mediator of the preconditioning effect, is the short period of ischaemia needed to induce the preconditioning effect. Skeletal muscle at rest is relatively resistant to ischaemia and in previous studies we have used microdialysis techniques to measure release of hypoxanthine from rat skeletal muscle in a model identical to that reported here. These data indicated that the release of substantial amounts of hypoxanthine, indicating breakdown of ATP, occurs only after ischaemia of three hours to resting rat skeletal muscle. These data appear to show that the time of ischaemia found to have a preconditioning effect is insufficient for significant breakdown of ATP in muscle as a potential source of adenosine precursors.

Several authors have indicated that a major site for the adenosine-mediated preconditioning effect in the heart is in the capillary endothelium. In the light of the arguments against skeletal muscle as a source of adenosine, it is tempting to speculate that local release of adenosine from ischaemic vascular smooth muscle, endothelial cells or blood cells may mediate the protective effects. In contrast to skeletal muscle, vascular endothelial tissue and vascular smooth muscle produce adenosine rapidly under hypoxia or ischaemia.
There appear to be a number of mechanisms by which adenosine may be protective against ischaemia/reperfusion injury to tissues including vasodilatation, prevention of leukocyte adherence and increases in the activity of antioxidant enzymes. In the data presented here adenosine prevented the rise in muscle myeloperoxidase activity indicating a reduced neutrophil sequestration within the reperfused muscle.

Adenosine has been reported to act to open ATP-dependant K⁺ channels in other tissues. This is thought to preserve ATP levels and have effects on ionic currents reducing the influx of calcium. It is also thought to exert its preconditioning effects in other tissues by interaction with either A₁ or A₃ receptors. Recent data appear to indicate that A₁ receptors may be involved in ischaemic preconditioning of skeletal muscle.

In conclusion, our data indicate that ischaemic preconditioning of skeletal muscle can be mimicked by the infusion of adenosine. It seems unlikely that adenosine is released from skeletal muscle cells sufficiently rapidly to mediate ischaemic preconditioning, but it may be released locally from cells of the microvascular network. Whatever the precise mechanism of action, the ischaemic preconditioning of skeletal muscle and treatment with adenosine or analogues warrants further investigation as a means of protecting skeletal muscle against reperfusion injury.

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References


Fig. 4

Content of HSP60 ( ), HSP72 ( ) and HSC70 ( ) in rat tibialis anterior muscles after reperfusion without pretreatment or with the preconditioning protocol of ischaemia for five minutes and reperfusion for five minutes. Data are median (75 percentile range), n = 6 animals for each treatment.