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Apoptotic Cell Death Makes a Minor Contribution to Reperfusion Injury in Skeletal Muscle in the Rat

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Objective: *to determine if apoptotic cell death contributes to skeletal muscle reperfusion injury.*
Methods: *leg ischaemia was induced in rats with a tourniquet and maintained for 4 h before reperfusion for 24 or 72 h. Apoptosis was assessed by morphology,* in situ *end labelling of DNA fragments, DNA laddering, expression of p53 mRNA and detection of caspase-3-like proteolytic activity.*
Results: *increased caspase-3-like activity was detected in muscle following ischaemia and zero, 24 h or 72 h of reperfusion. Levels remained relatively low but with a highly significant difference in enzyme activity between the ischaemic and non-ischaemic legs (p<0.0001, Repeated Measures Analysis of Variance). Morphological examination showed considerable oedema, disruption of muscle fibres and infiltration of white cells into tissues. Muscle nuclei did not show any morphological evidence of apoptosis and were negative for DNA fragmentation, while occasional neutrophils contained fragmented DNA. Expression of p53 was not induced by ischaemia and reperfusion and DNA ladders were not detected.*
Conclusions: *the cells undergoing apoptosis were infiltrating neutrophils rather than muscle cells and reperfused muscle was damaged largely by an inflammatory process involving considerable oedema.*

Key Words: Apoptosis; Reperfusion injury; Ischaemia; Caspases; Skeletal muscle.

Introduction

Reperfusion injury following lower extremity revascularisation is a common problem encountered in vascular surgery, the severity of which varies from minor leg swelling to compartment syndrome and multisystem organ failure. However, the available therapies are frequently ineffective and the biochemical and molecular mechanisms mediating this damage remain incompletely understood.

A number of mechanisms of damage have already been implicated in skeletal muscle reperfusion injury,[1] including generation of reactive oxygen species,[2] increased microvascular permeability,[3] release of lactate and potassium ions,[4,5] complement activation[6] and an increase in intracellular calcium[7,8] which can activate calcium-dependent proteases, leading to cellular injury. Activation of leukocytes which adhere to the endothelium and infiltrate the surrounding tissues also plays a vital role in the induction of tissue damage.[9,10]

Oxidative stress and the production of reactive oxygen intermediates can act as mediators of apoptosis.[11,12] The presence of reactive oxygen species generated by infiltrating neutrophils suggests that at least some of the damage to skeletal muscle may be mediated by apoptosis. Recent studies have demonstrated the involvement of apoptotic cell death in reperfusion injury in a range of tissues and different clinical situations. The characteristics of apoptotic cell death can be recognised in damaged neuronal cells following focal cerebral ischaemia[13] and reperfusion.[14] Similarly, brief periods of ischaemia followed by reperfusion in the kidney resulted in apoptotic cell death.[15] Ischaemia/reperfusion in cardiomyocytes[16,17] and in intact isolated rat hearts[18] also resulted in apoptosis. Using *in situ* end labelling of DNA strand breaks in autopsy specimens, apoptotic cell death was also recognised as a major mechanism of myocyte death during acute myocardial infarction in a clinical situation.[19,20] Apoptosis therefore appears to play a fundamental role in cellular damage occurring during reperfusion injury in a number of pathological settings and it would be likely that a similar mechanism of damage will occur in skeletal muscle.

The aim of this study was to test the hypothesis that apoptotic cell death is important in the pathogenesis of reperfusion injury in skeletal muscle by using a range of standard techniques to detect apoptosis. If apoptosis were to be identified as an important mechanism of cellular damage, this knowledge would point the way to new treatment modalities to interrupt the

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apoptotic cascade, thereby minimising damage to the ischaemic limb and to distant organs.

Materials and Methods

Rat model of ischaemia and reperfusion in skeletal muscle of the hind limb

Male Sprague–Dawley rats were anaesthetised by inhalation of halothane and oxygen. Body temperature was maintained at 37 °C using a heat lamp and the core temperature monitored with a rectal probe. The area over the left thigh was shaved, a photoplethysmography probe was attached to the skin over the femoral artery and blood flow was monitored on a Doppler ultrasound machine (Parks Medical Electronics; Aloha, Oregon, U.S.A.). To induce ischaemia, a tourniquet of fine nylon tape was tied tightly around the left thigh of the rat above the greater trochanter. Ischaemia was assessed to be complete if the Doppler trace remained flat and the foot appeared ischaemic. Ischaemia was confirmed every 15 min and maintained for 4 h before the tourniquet was released and the return of blood flow to the limb monitored by the return of the Doppler trace. The rats were then allowed to recover from anaesthesia and returned to the holding facility. To control for the effects of anaesthesia, sham-operated rats were treated identically to the ischaemic rats, except the tourniquet was not applied to the leg. The Animal Ethics Committees of The Queen Elizabeth Hospital and The University of Adelaide approved all experimental procedures used in this study.

Reperfusion was allowed to proceed for 0 (ischaemia only), 24 h or 72 h before the rats (four rats/time point) were sacrificed and tissues collected for analysis. The rats were anaesthetised with sodium pentobarbitone (Nembutal, 60 mg/kg), the chest opened and the animal perfused through the heart with 300 ml of normal saline to wash out blood elements. The washout was omitted from duplicate animals if the tissue was to be assessed by histological techniques. Skeletal muscle, consisting of a transverse block of the quadriceps muscle distal to the tourniquet was then collected and either snap-frozen in liquid nitrogen or fixed in 10% buffered formalin for further analysis.

DNA fragmentation

Fresh tissue was pulverised under liquid nitrogen, suspended in STE buffer (100 mM NaCl, 10 mM Tris-HCl, pH 8.0; 1 mM EDTA) plus 1% SDS and digested overnight at 37 °C with 50 μg/ml proteinase K plus 10 μg/ml RNaseA (Roche Molecular Biochemicals, Mannheim, Germany). DNA was isolated by standard phenol/chloroform extraction and ethanol precipitation techniques, electrophoresed through 1% agarose gels containing 50 μg/ml ethidium bromide and examined under UV light for the presence of DNA ladders.

In situ *end labelling for DNA fragmentation (TUNEL assay)*

Paraffin sections of skeletal muscle were analysed for the presence of apoptotic nuclei using the *in situ* cell death detection kit (Roche Molecular Biochemicals). Tissue sections were dewaxed and then rehydrated by placing the slides in decreasing concentrations of ethanol in water. Proteinase K (20 μg/ml in 10 mM Tris-HCl) was applied to each section for 15 min at room temperature, then the slides were placed in a bath of phosphate-buffered saline (PBS). Endogenous peroxidase activity was quenched by incubating the slides in 3% hydrogen peroxide in water for 5 min at room temperature. The manufacturer's instructions were followed for the remainder of the protocol, except that sections were counter-stained in Harris' haematoxylin (Sigma Aldrich, St. Louis, MO, U.S.A.), rather than methyl green.

A negative control slide was included for each tissue section by omitting the terminal deoxynucleotidyl transferase enzyme. A positive control consisted of a section of normal rat skeletal muscle treated with DNase I to induce nicks in the nuclear DNA. After proteinase digestion, the section was incubated with DNase buffer (0.1 M sodium acetate, 5 mM $MgSO_4$, pH 5.0) for 2 min at room temperature, then with 10 μg/ml of DNase I (Roche Molecular Biochemicals) in DNase buffer for 10 min. The slide was rinsed extensively with distilled water and processed with the other slides.

Caspase-3-like proteolytic activity

Caspase-3-like activity was determined as described in Medina *et al.*,[21] using a synthetic peptide substrate, z-aspartic acid-glutamic acid-valine-aspartic acid-7-amino-4-trifluoromethyl coumarin (Z-DEVD-AFC) (Kamiya Biomedical Co., Tukwila, Wa, USA). Total protein was isolated by pulverising frozen tissue with a mortar and pestle and the resultant powder resuspended in lysis buffer (5 mM Tris-HCl, 5 mM EDTA,

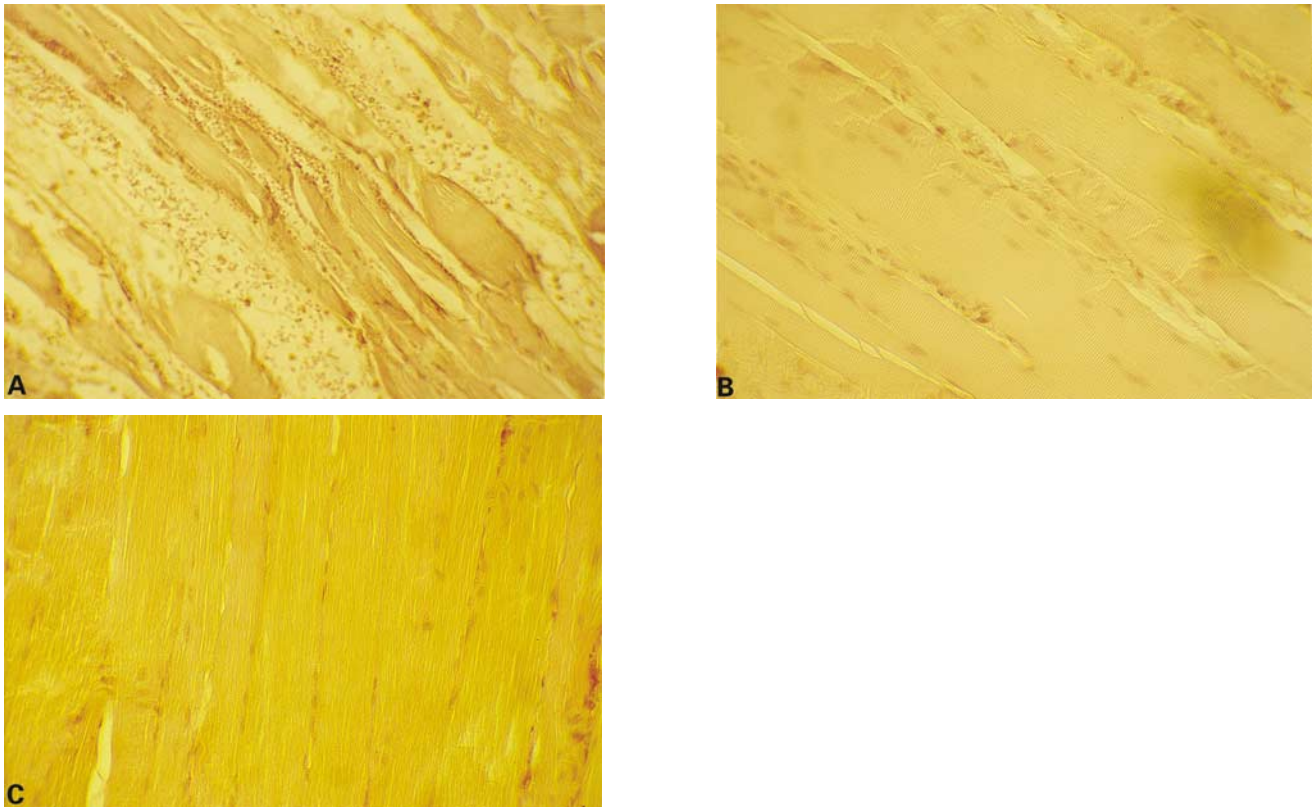

Fig. 1. The rat was subjected to 4 h ischaemia and 24 h or 72 h reperfusion before muscle tissues were harvested and paraffin sections stained with haematoxylin and eosin (50 × magnification). (A) Muscle from limb subjected to 4 h ischaemia and 24 h reperfusion. (B) Muscle from limb subjected to 4 h ischaemia and 72 h reperfusion. (C) Muscle from contralateral control limb.

0.5% NP-40, pH 7.5). Lysates were centrifuged at 12 000 g for 15 min, supernatants snap-frozen in liquid nitrogen and stored at −80 °C for subsequent analysis. Protein concentrations were determined by the Bradford Protein Microassay procedure (Bio-Rad Life Sciences, Hercules, CA, U.S.A.).

Briefly, 100 µg of protein were mixed with 8 µM of the synthetic caspase substrate in a total volume of 1 ml of protease buffer (50 mM Hepes, 10% sucrose, 10 mM dithiothreitol, 0.1% CHAPS, pH 7.4) and incubated at room temperature. Fluorescence was measured at 30 min intervals over 7 h and at 24 h, using a Perkin Elmer LS50 spectrofluorimeter with excitation wavelength of 400 nm, emission wavelength of 505 nm and slit width of 10 nm. Two human malignant cell lines were used as positive controls for the assay of caspase-3-like activity, Jurkat, derived from an acute T lymphoblastoid leukemia and LIM1215 colon carcinoma.[22] Both cell lines (106 cells) were treated with sodium butyrate (4 mM) for 16 h to induce activation of caspases[21] and caspase-3-like activity assayed as described above (data not shown).

Fluorescence measurements were analysed by using an unbalanced Repeated Measures Analysis of Variance with factors to allow for change over time and to allow for change over time to be dependent on treatment (normal, sham-operated or ischaemia/reperfusion), and duration of reperfusion. Data from all animals that underwent the operative procedures were pooled and the levels of caspase-3-like activity in the ischaemic legs (12 legs) were compared with that in the non-ischaemic legs (36 legs) using Repeated Measures Analysis of Variance). Program 5V from the BMDP statistical software package was used for statistical analysis (Ed: W. Dixon, UCLA).

Expression of p53 mRNA by Northern blotting

A 265 bp partial cDNA probe coding for human p53 was amplified by PCR techniques from cDNA synthesised by reverse transcription from the T47D human breast carcinoma cell line. The identity of the PCR product was confirmed by direct sequencing. Since homology between rat and human p53 was 93% over the defined section of the cDNA, the human p53 cDNA probe had adequate homology to detect rat p53 mRNA by Northern blotting.

Total cellular RNA was isolated from tissues by

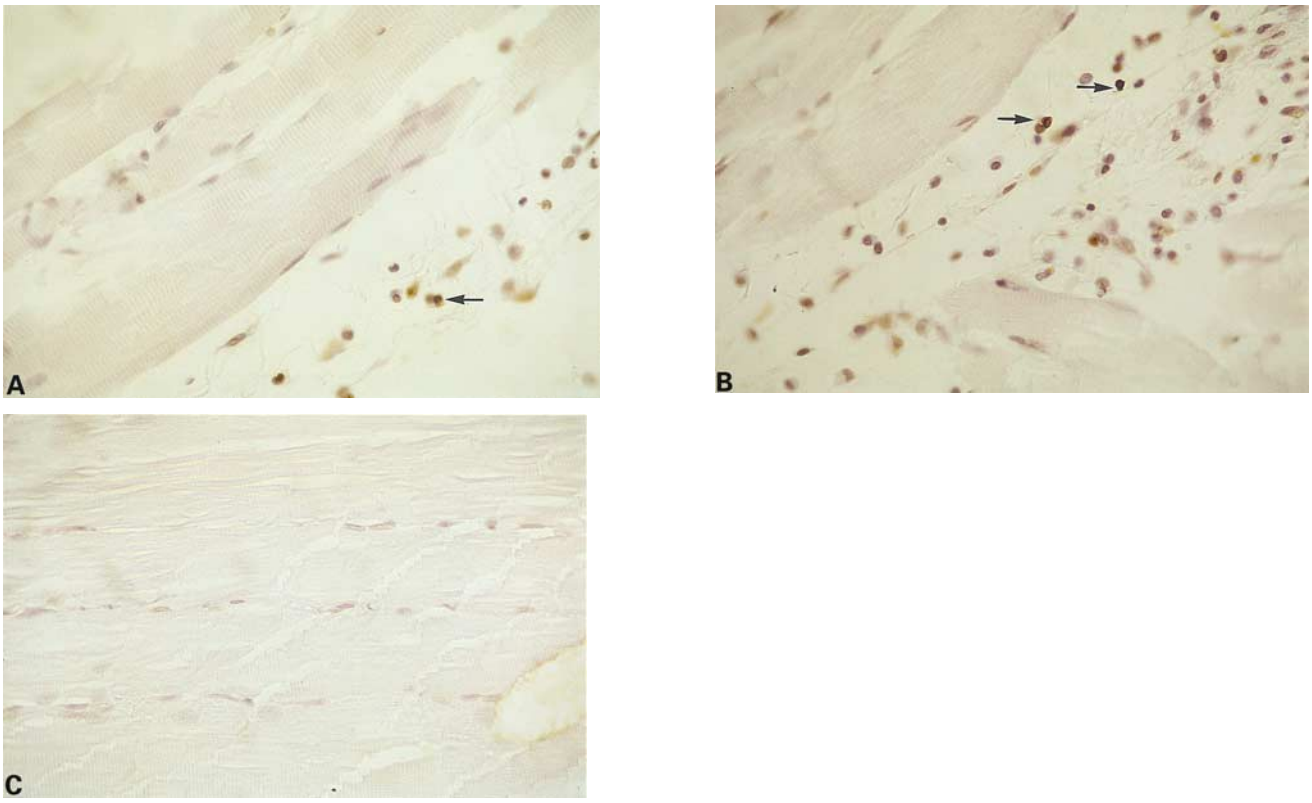

Fig. 2. The rat was subjected to 4 h ischaemia and 24 h reperfusion before muscle tissues were harvested and *in situ* DNA fragmentation detected by the TUNEL technique (200× magnification). Arrows denote TUNEL-positive nuclei in neutrophils that also display an apoptotic morphology. (A, B) Muscle from limb subjected to 4 h ischaemia and 24 h reperfusion. (C) Muscle from contralateral control limb.

standard guanidinium thiocyanate lysis techniques[23] and 10 μg were electrophoresed through 1% agarose/16% formaldehyde gels before transfer to Genescreen Plus nylon membrane. The p53 cDNA probe was labelled with ^{32}P-dATP and hybridised overnight before washing and exposure to X-ray film. As a positive control on the Northern blots, RNA was isolated from the MCF7 human breast carcinoma cell line, which expresses high levels of p53.

Results

Histological analysis

Histological examination of the treated muscle following 4 h ischaemia and 24 h reperfusion showed a major influx of inflammatory cells, mainly neutrophils and marked disruption of the muscle fibres, accompanied by considerable oedema (Fig. 1A). This confirmed that the ischaemic insult was effective in causing considerable damage to the muscle. The nuclei of some of the neutrophils showed the characteristic appearance of apoptotic bodies, but all muscle nuclei remained intact and showed no evidence of apoptosis. Following 72 h of reperfusion, disruption of muscle fibres was less evident, suggesting a reduction in the degree of oedema in the tissues (Fig. 1B). In comparison, the muscle of a sham-operated rat appeared undamaged (Fig. 1C).

In situ *end labelling for DNA fragmentation (TUNEL assay)*

The presence of cells undergoing apoptotic cell death was assessed by *in situ* end-labelling of DNA fragments (TUNEL technique). Occasional TUNEL-positive neutrophils were observed in sections of the treated muscle (4 h ischaemia and 24 h reperfusion) which were infiltrating the disrupted muscle fibres but all muscle nuclei were TUNEL-negative (Figs 2A and 2B). Some of the TUNEL-stained nuclei showed the characteristic morphological appearance of apoptotic bodies (Figs 2A and 2B, shown by arrows). There was no evidence of TUNEL-positive apoptotic cells in the contralateral limb or in sham-operated animals (Fig. 2C). A positive control slide consisting of normal

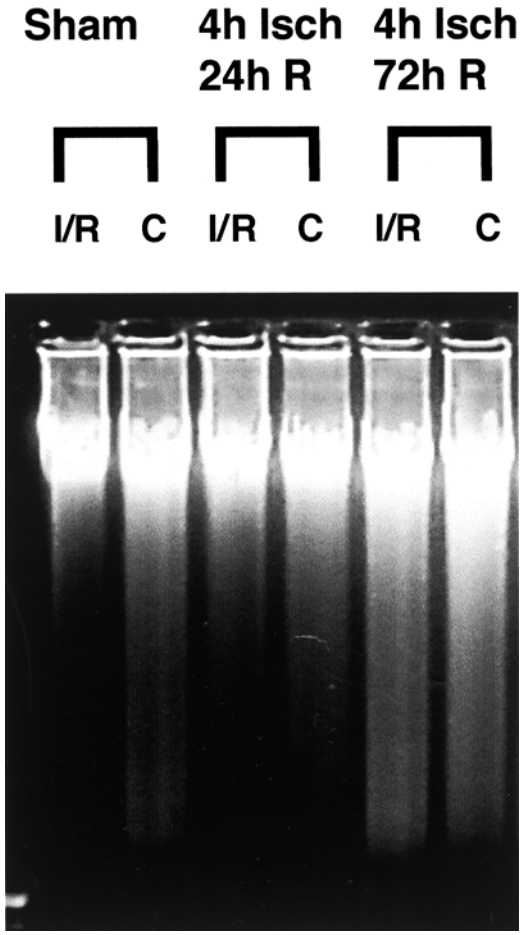

Fig. 3. DNA was isolated from muscle from rats treated with 4 h ischaemia and varying times of reperfusion and analysed on 1% agarose gels for the presence of DNA ladders. The first two tracks show DNA isolated from left and right legs of a sham-operated animal. I/R: Muscle from leg subjected to 4 h ischaemia and 4 h or 24 h reperfusion. C: control contralateral limb.

skeletal muscle treated with DNase1 was highly-positive with the majority of nuclei staining darkly (data not shown).

DNA fragmentation

DNA was isolated from ischaemic, contralateral and sham-operated muscles and assessed on agarose gels for the presence of DNA ladders characteristic of apoptosis. DNA ladders were not detected in skeletal muscle that had undergone 4 h ischaemia and varying duration of reperfusion from 24 h–72 h (Fig. 3). Similarly, no ladders were detected in DNA isolated from skeletal muscle from sham-operated rats.

Caspase-3-like activity

The activation of proteolytic activity of the caspase enzymes is recognised as being integral to the execution phase of apoptotic cell death.[21] The rate of release of the fluorescent AFC tag from the synthetic peptide was determined over time as a measure of apoptotic activity. In a preliminary analysis, there was no difference in caspase-3-like proteolytic activity between the contralateral limbs and limbs from the sham-operated animals (data not shown). For the final analysis, these two groups were therefore pooled. At 1 h, ischaemic legs contained 7.79 ± 0.34 fluorescence units and non-ischaemic legs, 6.98 ± 0.32. After 6 h of release of AFC, ischaemic legs were assayed to produce 31.23 ± 1.08 fluorescence units and the non-ischaemic control legs; 26.40 ± 0.60. Early in the assay time there was little difference between the two mean values, but this difference became larger as time increased to being approximately 5 units at 6 h. This difference in fluorescence units at 6 h was highly statistically significant ($p<0.0001$). There was no significant difference in caspase-3-like activity in muscle tissues that had undergone either 0, 24 h or 72 h reperfusion.

Expression of p53 mRNA by Northern blotting

Expression of the pro-apoptotic gene, p53, is induced during reperfusion injury in the brain.[24] Northern blotting failed to demonstrate any induction of expression of p53 mRNA following ischaemia/reperfusion either in the skeletal muscle or in the lungs of rats subjected to ischaemia (data not shown).

Discussion

In the current study we did not detect any evidence that the muscle cells themselves were undergoing apoptotic cell death. Morphological examination showed that the muscle nuclei remained intact with no evidence of the formation of apoptotic bodies. There was also no evidence of apoptosis of endothelial cells. Similarly, muscle nuclei were all negative for DNA fragmentation by TUNEL staining, and no laddering could be detected when DNA isolated from muscles was analysed on agarose gels. In agreement with this data, no induction of expression of the pro-apoptotic gene, p53 could be detected.

There was considerable infiltration of neutrophils into the muscle tissues of the ischaemic leg following

24 h reperfusion. The nuclei of a small proportion of the neutrophils showed evidence of apoptosis, both morphologically and by positive TUNEL staining. In agreement with this data, a low but significant increase in caspase-3-like proteolytic activity was detected in muscle isolated from legs that had undergone 4 h ischaemia and variable lengths of reperfusion, when compared to the contralateral non-ischaemic and sham-operated legs. It is likely that the cells that contain activated caspase-3-like proteases are the tissue-infiltrating neutrophils. No significant differences in the levels of caspase-3-like activity could be detected between legs that had undergone ischaemia alone, compared to legs that had been reperfused for 24 h or 72 h.

It has been suggested that, since myocytes are terminally differentiated and have exited the cell cycle, they are relatively more resistant to apoptosis.[25] In agreement with this suggestion, ARC, an inhibitor of caspase-mediated apoptosis, is expressed almost exclusively in human cardiac and skeletal muscle.[26] However, under appropriate conditions myocytes are capable of undergoing apoptotic cell death. Apoptosis of skeletal muscle cells has been demonstrated in muscular dystrophies,[27] during hind limb unloading-induced atrophy[28] and during exercise-induced damage,[29,30] demonstrating that the myocytes contain the necessary patterns of gene expression to be capable of undergoing apoptosis when the appropriate pro-apoptotic trigger is applied to the cells. Our inability to detect apoptosis in skeletal muscle may reflect a difference in susceptibility between cardiac and skeletal myocytes, possibly due to differences in the relative numbers of fast and slow twitch fibres, since the latter are more susceptible to apoptosis.[31] The tissue assayed in the current study was taken from the entire quadriceps muscle and would have contained a mixture of both fibre types.

It is possible that, in the current study, the duration of either ischaemia or reperfusion was not optimal to induce apoptosis. In preliminary experiments (data not shown), the hind limb of rats was subjected to 2 h of ischaemia and variable duration of reperfusion from 2 h to 72 h, but no detectable damage was observed upon histological examination. It was therefore decided that longer ischaemia was required to induce damage and the current study used 4 h of ischaemia, also commonly used by other investigators.[3,10] It was not possible to maintain to ischaemia for longer than 4 h, as, due to ethical concerns, longer duration anesthetics were not administered to the rats. The rat model of ischaemia/reperfusion is in common use and results using this methodology have been published by a number of investigators.[10,32] Similarly, the assays of apoptosis are also those that are widely accepted. It would therefore appear unlikely that our inability to detect significant muscle apoptosis is due to technical difficulties. Clinically, the severity of acute limb ischaemia is quite variable but is frequently "total" or "near-total" following acute embolisation or trauma. The model of reperfusion injury used in this study thus has clinical relevance as well as being well-validated in previous studies.

The current study is in agreement with a recently-published report by Knight *et al.*,[33] which reported that the mechanisms of cellular damage following skeletal muscle ischaemia and reperfusion could largely be attributed to necrosis rather than apoptosis. Using a similar technique of inducing ischaemia in the hind-limb of a rat with a tourniquet, they were not able to demonstrate any apoptosis of the skeletal muscle cells. A small number of TUNEL-positive endothelial and smooth muscle cells were observed early in reperfusion, followed later by the appearance of apoptotic neutrophils. Their methods of detection of apoptosis relied largely on morphological analysis. In the current study we have extended the scope of the apoptotic assays used by also examining DNA ladders, expression of p53 mRNA and induction of caspase-3-like proteolytic activity.

In conclusion, the demonstration that apoptosis does not play a major role in the damage to skeletal muscle during reperfusion injury has excluded a number of therapeutic interventions which could have potentially reduced the severity of damage. Other, as yet unidentified, mechanisms of damage to muscle and to distant organs will therefore need to be targeted for the development of clinically-useful treatments for skeletal muscle reperfusion injury.

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