Dantrolene sodium protects against experimental ischemia and reperfusion damage to skeletal muscle

Leslie Klenerman[1], Nicola M Lowe[2], Ian Miller[2], Patricia R Fryer[3], Colin J Green[4] and Malcolm J Jackson[2]

The effect of 4 hours of ischemia followed by reperfusion for 1 hour has been studied in fully anesthetized rabbits. Muscles from the limb subjected to ischemia and reperfusion showed considerable ultrastructural damage, although the distribution of damage between muscles was not uniform (anterior tibialis > soleus > quadriceps).

Damage to the muscle was associated with a significant increase in the concentration of some indicators of free radical-mediated processes (thiobarbituric acid-reactive substances and diene conjugates), although others (glutathione and protein sulfhydryl groups) were unchanged. Reperfused muscles also showed considerable changes in their calcium and sodium contents.

Treatment of animals with dantrolene sodium (4 mg/hr) throughout the periods of ischemia and reperfusion was found to preserve the ultrastructural appearance of quadriceps, soleus and anterior tibialis muscles. No effect of dantrolene sodium on indicators of free radical activity or muscle cation content was seen.

Departments of [1]Orthopaedic and Accident Surgery and [2]Medicine, University of Liverpool, P.O. Box 147, Liverpool, L69 3BX, U.K.; [4]Section of Surgical Research, and [3]Electron Microscopy, Northwick Park Research Centre, Watford Road, Harrow, Middlesex, HA1 3NJ, U.K. Correspondence: Professor M.J. Jackson. Tel +44 51-706 4077. Fax -706 5802
Submitted 94-08-10. Accepted 95-03-28

The biochemical mechanisms underlying ischemia/reperfusion damage to tissues have been the subject of a large number of recent studies. In particular, oxidizing free radical species have been implicated in this process (McCord 1985). Muscle tissue has been thought to be relatively insensitive to ischemia/reperfusion-induced damage, but a number of recent papers have suggested that oxygen-free radical species may be produced during reperfusion of skeletal muscle (Lindsay et al. 1989, Pang 1990, Walker 1991) and are mediators of the resulting damage (Perler et al. 1990, Oredsson et al. 1991).

Other data concerning the mechanisms underlying damage to skeletal muscle induced by a number of different stresses have suggested that an accumulation of intracellular calcium may be a key step in the process (Jones et al. 1984, McArdle et al. 1992). This calcium accumulation causes damage to the plasma membrane via an activation of phospholipase A2 (Jackson et al. 1984).

In order to clarify the nature of the structural damage to skeletal muscle induced by ischemia and reperfusion and the biochemical changes which occur parallel to these, a model of limb ischemia in the rabbit has been developed. To define the role of disturbances in muscle calcium homeostasis in the damage seen post-reperfusion of ischemic muscle, dantrolene sodium has been utilized to influence the release of calcium from the sarcoplasmic reticulum (Morgan and Bryant 1977). Dantrolene sodium is used as a skeletal muscle relaxant for the symptomatic relief of spasticity (Ellis and Carpenter 1972), as a specific drug in preventing and treating malignant hyperthermia (Gronert 1980) and as a possible therapeutic agent in exertional muscle pain syndromes (Bertorini et al. 1982, Haverkort-Poels et al. 1987).

Animals and methods

New Zealand white rabbits (weight approximately 2.5 kg) maintained on a standard laboratory feeding regimen were used. All animals were fully anesthetized with fentanyl-fluanisone (0.3 mL/kg Hypnorm®) and diazepam (2.5 mg/kg Valium 20®), intubated and mechanically ventilated throughout the experiment. The animal was placed on a thermostatically-controlled heating blanket, the thighs and legs were shaved and rectal temperature was monitored.

A 5 cm pneumatic tourniquet cuff was applied to one thigh as high as possible, the limb was exsanguinated and the tourniquet was inflated to approximately 300 mmHg for 4 hours. A heating coil was placed around the ischemic limb to maintain a skin temperature of 35 °C. After 4 hours, the cuff was deflated and reperfusion was allowed to occur for 1 hour. The animals were then killed and muscle samples from the soleus, anterior tibialis and quadriceps (distal to the tourniquet) from both legs were removed and small strips fixed in glutaraldehyde for electron microscopy. The rest of the muscle samples were rapidly frozen in liquid nitrogen for biochemical analysis. 11 animals were studied in these experiments.

Where dantrolene sodium (Norwich Eaton Pharmaceuticals, U.K.) was used (5 animals), it was administered throughout the experiment by infusion into an ear vein at a dose of 4 mg/hr.

Electron microscopy

Primary fixation was in 3% glutaraldehyde in 0.1M phosphate buffer pH 7.4 for 2 hrs at room temperature. After washing in the same buffer, postfixation was in 1% osmium tetroxide in the same buffer for 1 hr. After a further buffer wash, dehydration was carried out in a graded acetone series. The tissue was embedded in Spurr's low-viscosity resin. Ultrathin sections were collected on uncoated copper grids, stained with aqueous uranyl acetate and lead citrate before viewing in a Philips 300 or a JEOL 1200 EX electron microscope. All electron microscope reagents were obtained from Agar Scientific (Stansted, Essex).

Indicators of free radical activity

The thiobarbituric acid reactivity (TBARS) of muscle samples was assayed following incubation of homogenate at 37 °C for 1 hour (Jackson et al. 1983). Diene conjugates were assessed by examining their UV absorbance at 234 nm, as described by Rechnagel and Ghoshall (1975).

The total and oxidized glutathione contents of muscles was analyzed by the glutathione reductase recycling method (Anderson 1985) with derivitization, using 2-vinylpyridine prior to analysis of the oxidized form. Muscle protein thiol groups were analyzed by titration of the sulfosalicylic acid precipitate from the glutathione assay with 5,5-dithiobis-2-nitrobenzoic acid essentially as described by Di Monte et al. (1984).

Cation content of muscles

Muscle samples were freeze-dried and analyzed for calcium, magnesium, potassium and sodium contents by atomic absorption spectrophotometry (Jackson et al. 1985).

^{99m}Technetium uptake

Animals were given an intravenous injection of ^{99m}Tc-pyrophosphate in isotonic saline (1 mL containing 50 MBq ^{99m}Tc) via an ear vein at the end of the tourniquet period. Blood serum and samples of muscles from both hind limbs were analyzed for ^{99m}Tc by γ-counting and the ^{99m}Tc content of muscle samples expressed in terms of μL equivalents of serum.

Reperfusion of muscles

An estimate of the amount of reperfusion of individual muscles was obtained by injection of 0.5 mL ^{125}I-albumin (Amersham International) 5 min before the animals were killed. The ^{125}I contents of serum and muscle samples were then analyzed by γ-counting and the blood content of the muscles calculated.

Statistics

Statistical analysis was undertaken by paired or unpaired Student's t-tests, as appropriate. Where data were non-gaussian in distribution (assessed by the Shapiro-Wilk test), the Mann-Whitney/Wilcoxon rank sum test was applied.

Results

Nature of the damage induced by reperfusion

All the muscles subjected to ischemia and reperfusion showed substantial evidence of damage. Changes were considerably greater in the anterior tibialis muscle than in the other two. Damage did not appear to be limited to any particular organelle or structure and was present throughout the tissue. Dantrolene sodium had a partially protective effect against this damage, particularly in the anterior tibialis muscle (Table 1). These effects were mainly evident concerning disruption of the myofibrillar structure and membrane damage induced by the ischemia/reperfusion (Figure 1). No effect of dantrolene sodium was seen on the normal appearance of the untreated contralateral muscles. ^{99m}-Technetium pyrophosphate uptake was higher ($p < 0.05$) in reperfused anterior tibialis muscles than in the control (Table 2) and there was a tendency for the uptake of the isotope to be higher in the other muscles. Dantrolene sodium tended to reduce the mean uptake of this isotope in the reperfused anterior tibialis muscles, but this did not reach statistical significance.

Table 1. Summary of ultrastructural changes in control and reperfused muscles

Treatment	Mitochondria	Glycogen	Muscle fibers	Membranes
Soleus				
Control	Normal	Abundant	Normal	Intact
Reperfused	Swollen Disrupted	Sparse	Variably damaged	Distended
Reperfused-dantrolene treated	Variable changes	Variable depletion	Some damage	Some damage
Quadriceps				
Control	Normal	Normal	Normal	Intact
Reperfused	Normal	Variable	Variably damaged	Some vesiculation
Reperfused-dantrolene treated	Normal	Normal	Normal	Intact
Anterior tibialis				
Control	Normal	Abundant	Mostly normal	Intact
Reperfused	Swollen and disrupted	Sparse	Severely damaged	Severely damaged
Reperfused-dantrolene treated	Swollen and disrupted	Variable depletion	Generally normal	Generally normal

Table 2. ^{99m}-Technetium content of muscles in µL of serum equivalents. Mean, *SEM*

	Untreated animals (n 6)		Dantrolene-treated animals (n 5)	
	Control leg	Reperfused leg	Control leg	Reperfused leg
Soleus	125 *6.7*	318 *30*	98 *1.5*	383 *26**
Quadriceps	59 *0.2*	138 *41*	57 *2.5*	75 *6.8*
Anterior tibialis	151 *24*	398 *30**	*83 8.2*	237 *20**

* Value different from control leg in same group of animals, $p < 0.05$.

Table 3. Total muscle glutathione, oxidized glutathione and protein sulfhydryl groups in muscles presented as µmol/g protein. Mean, *SEM*. (n 6)

	Quadriceps		Soleus		Anterior tibialis	
Total glutathione						
Control	7.2	*0.5*	15.1	*1.3*	7.0	*0.4*
Reperfused	7.6	*0.4*	11.7	*1.7*	6.4	*0.6*
Oxidized glutathione						
Control	0.72	*0.14*	0.64	*0.06*	0.64	*0.15*
Reperfused	0.76	*0.21*	0.61	*0.12*	0.80	*0.20*
Protein sulfhydryl groups						
Control	0.033	*0.007*	0.045	*0.006*	0.031	*0.005*
Reperfused	0.038	*0.004*	0.040	*0.007*	0.049	*0.008*

Extent of reperfusion of the muscles

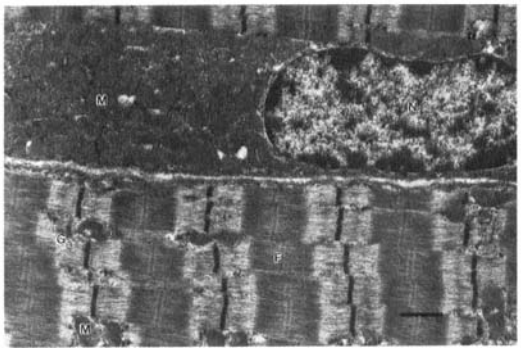
There was no evidence of a significant underperfusion or hyperemia in the previously ischemic muscles. Results expressed as equivalents of serum were control soleus: 7.0, *2.9* (mean, *SEM*), reperfused soleus 9.0, *7.4;* control quadriceps: 3.7, *2.4;* reperfused quadriceps 3.5, *1.1;* control anterior tibialis: 4.6, *2.5;* reperfused anterior tibialis 4.4, *4.0*.

Indicators of free radical activity

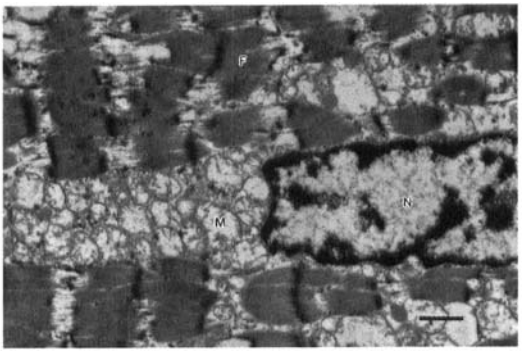
No differences were found between the total glutathione, oxidized glutathione or protein sulfhydryl contents of reperfused and control muscles (Table 3).

The TBARS and diene conjugate contents of the ischemic/reperfused and control muscles are presented in Tables 4 and 5. The reperfused anterior tibialis muscles showed an elevation of both TBARS ($p < 0.05$) and diene conjugates ($p < 0.05$), but no differ-

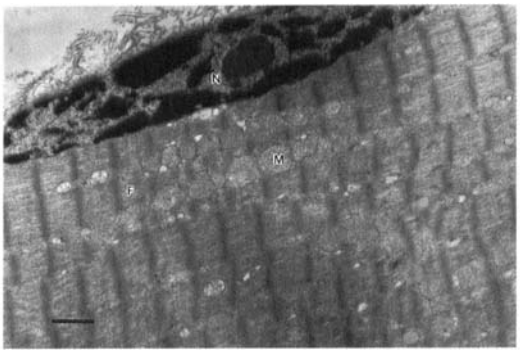
Figure 1. Example of electron micrographs of muscles subjected to a period of ischemia and reperfusion with or without treatment with dantrolene sodium. Abbreviations: M mitochondria, F muscle fiber, G glycogen, N nucleus. Bar 1 µm.



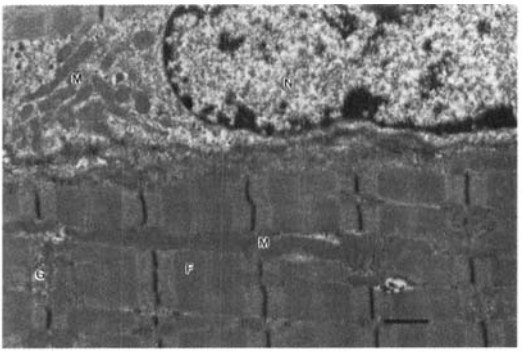
Control soleus muscle, magnification 15,000



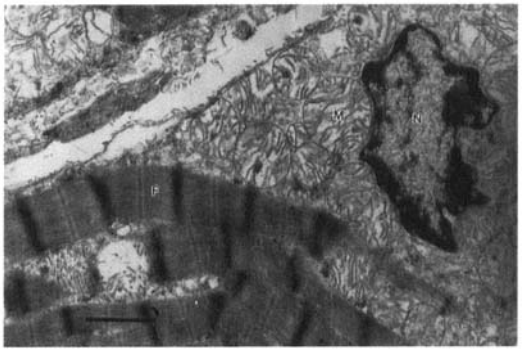
Reperfused soleus muscle from an untreated animal, magnification 15,000, showing enlargement and disruption of mitochondria, disruption of the myofibrillar structure and disorganization of the nucleus.



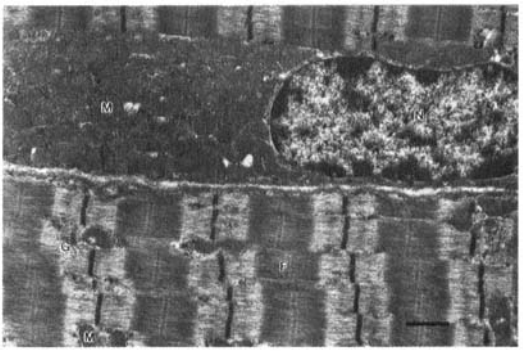
Reperfused soleus muscle from a dantrolene-treated animal, magnification 15,000, showing mitochondria which mainly retain a normal appearance, partial restoration of the myofibrillar structure and a relatively intact nucleus.



Control anterior tibialis muscle, magnification 15,000.



Reperfused anterior tibialis muscle from an untreated animal, magnification 23,000, showing severe disruption of myofibrillar, mitochondrial and nuclear structure.



Reperfused anterior tibialis muscle from a dantrolene-treated animal, magnification 15,000, showing generally normal nuclear and myofibrillar structure, but grossly enlarged and disrupted mitochondria.

ences were seen in other muscles. Dantrolene sodium treatment had no effect on either of these indicators of free radical activity.

The data presented also demonstrate that the control highly oxidative soleus muscle of the rabbit contains greater amounts of glutathione, TBARS and diene conjugates than the other two muscles studied (Tables 3, 4, and 5).

Table 4. Thiobarbituric acid-reactive substances (TBARS) in muscles as A_{532}/gm muscle. Mean, *SEM*

	Untreated animals (n 6)		Dantrolene-treated animals (n 5)	
	Control leg	Reperfused leg	Control leg	Reperfused leg
Soleus	13.5 *0.6*	11.0 *0.3*	13.5 *0.9*	11.6 *1.0*
Quadriceps	4.9 *0.3*	4.6 *0.3*	4.4 *0.3*	5.6 *0.4*
Anterior tibialis	5.6 *0.3*	10.8 *0.6**	7.9 *0.4*	10.0 *0.9*

* Different from control leg in same group of animals, $p < 0.01$.

Table 5. Diene conjugate content of muscles as A_{234}/g muscle. Mean, *SEM*

	Untreated animals (n 6)		Dantrolene-treated animals (n 5)	
	Control leg	Reperfused leg	Control leg	Reperfused leg
Soleus	0.77 *0.02*	0.73 *0.02*	0.69 *0.02*	0.97 *0.03**
Quadriceps	0.53 *0.01*	0.56 *0.02*	0.54 *0.01*	0.65 *0.01*
Anterior tibialis	0.56 *0.02*	0.78 *0.02**	0.70 *0.02*	0.75 *0.04*

* Different from control leg in the same group of animals, $p < 0.02$.

Table 6. Calcium and sodium contents of rabbit muscles as µmol/g dry weight. Mean, *SEM*

	Untreated animals (n 6)		Dantrolene-treated animals (n 5)	
	Control leg	Reperfused leg	Control leg	Reperfused leg
Calcium				
Soleus	4.9 *0.7*	10.9 *2.6*[a]	2.5 *0.5*[b]	10.4 *3.0*[a]
Quadriceps	3.6 *0.3*	3.1 *0.4*	2.2 *0.3*[b]	2.2 *0.6*
Anterior tibialis	3.3 *0.3*	5.2 *0.6*[a]	2.3 *0.4*[b]	4.2 *1.0*
Sodium				
Soleus	122 *20*	288 *47*[a]	114 *20*	161 *30*[c]
Quadriceps	70 *6.5*	102 *41*	82 *13*	83 *8.6*
Anterior tibialis	90 *18*	176 *27*[a]	76 *18*	127 *10*

[a] Values different from control leg in same group of animals.
[b] Values different from control leg in untreated animals.
[c] Value different from reperfused leg in untreated animals.
$P < 0.05$.

Cation content of muscles

Major changes in muscle ion homeostasis were caused by the period of ischemia and reperfusion. In particular, the calcium and sodium (Table 6) contents of the reperfused soleus and anterior tibialis muscles were elevated compared to the control, whereas the reperfused quadriceps muscle was not affected.

Dantrolene sodium failed to reverse the increase in muscle calcium content seen in the reperfused anterior tibialis and soleus muscles, but it reduced the sodium content of the reperfused soleus muscles (Table 6).

Reperfusion also caused a fall in the magnesium (control: 21.1, *2.3* cf. reperfused: 14.1, 2.1 µmol/g) and potassium (control: 177, *18.3* cf. reperfused: 99, *16.1* µmol/g) contents of the soleus muscle, which was not affected by dantrolene sodium treatment.

Discusson

The experimental model utilized here has been shown to provide reproducible evidence of ischemia/reperfusion-induced damage to skeletal muscle. In preliminary (unpublished) experiments, we determined that

4 hours of ischemia followed by 1 hour of reperfusion to the hind limb provides an amount of damage to the muscle which is reproducible and is reasonably realistic in terms of the length of time the acutely ischemic human limb may be subjected to loss of blood supply during complicated orthopedic surgery or limb reattachment. These preliminary experiments also demonstrated that the ischemic rabbit hind limb showed a much greater fall in temperature than a human limb under equivalent circumstances and hence the rabbit limb was maintained at 35 °C throughout the study described here.

It is not clear why the anterior tibialis muscle should be relatively more affected by the ischemia and reperfusion than the other muscles, since all were subjected to the same length of ischemia and appeared to fully reperfuse. The anterior tibialis muscle of the rabbit is composed primarily of type II fibers as also is the quadriceps, in comparison to the highly oxidative (type I) soleus muscle. Differences in fiber type may therefore play a role.

The possibility that free radical-mediated reactions may be important in the damage seen following an ischemic episode in several tissues has been discussed for some time (McCord 1985). Recently, a number of authors have examined the role of free radical species in ischemic damage to skeletal muscle (Lindsay et al. 1989, Pang 1990, Perler et al. 1990, Oredsson et al. 1991, Walker 1991). The data presented here indicate that no gross oxidation of the major intracellular thiols (Table 3) has occurred in the reperfused tissues, but the two indicators of lipid peroxidation (TBARS and diene conjugates) were elevated in the reperfused anterior tibialis muscles (Tables 4 and 5). The elevated indicators of lipid peroxidation were therefore found in the tissue with the greatest amount of damage (Table 1), with no evidence of elevated indicators of free radical activity in the soleus muscle where some damage, particularly to the mitochondria, was apparent (Table 1, Figure 1). There is therefore the possibility that the increases in lipid peroxidation products seen in the reperfused anterior tibialis muscles may be secondary to the gross damage seen therein. Halliwell and Gutteridge (1985) have suggested that damaged tissue can produce free radicals and this explanation of our results cannot be ruled out on the basis of the data available.

Since the changes observed in the mitochondria of the reperfused muscles are similar to those produced by experimental damage due to cellular calcium overload (Duncan et al. 1979) and the accumulation of ^{99m}-technetium pyrophosphate suggested that calcium might be accumulating in the damaged muscles, the cation content of the muscle was examined (Table 6). Total muscle calcium was elevated in both the reperfused soleus and anterior tibialis muscles, in association with a rise in sodium and some fall in potassium and magnesium. Such changes indicate a loss in the ability of membranes to maintain ion gradients. An elevation of intracellular calcium can cause substantial damage to muscle tissue (Wrogemann and Pena 1976, Jones et al. 1984) and it has been implicated in the mechanisms of damage to skeletal muscle which occurs in a number of different situations, such as following excessive contractile activity (Jones et al. 1984), in dystrophic muscle (Jackson et al. 1985) and in malignant hyperthermia (Gronert 1980).

Dantrolene sodium is known to reduce the release of calcium from muscle sarcoplasmic reticulum (Morgan and Bryant 1977) and this is thought to be its mode of action in the treatment of malignant hyperthermia (Gronert 1980). Analysis of the total calcium content of the dantrolene-treated muscle indicated no reduction in total muscle calcium content in the muscles, although there was evidence for the protection of their ultrastructure. There are several possible explanations for this apparent paradox. The dantrolene may have helped to maintain more of the increased muscle calcium within the sarcoplasmic reticulum stores, and thus to maintain a low free intracellular calcium in the muscle cytosol with no change in total muscle calcium. Alternatively, dantrolene may have acted by maintaining compartmentalization of the calcium in other stores, such as mitochondria, as proposed by Haverkort-Poels et al. (1987). The only potentially relevant biochemical effect of dantrolene observed was a reduction in muscle sodium content in the reperfused soleus muscle. We are unaware of any data suggesting that dantrolene can influence muscle sodium handling or that sodium plays any role in the genesis of muscle damage, independently of calcium. It may therefore be that the changes in muscle sodium content reflect a reduction in the amount of damage which occurred in the soleus muscle.

Our studies have examined only the possible inhibitory effect of dantrolene sodium on muscle damage when the drug was given throughout the experiment. Infusion of the drug during the ischemic period was probably unnecessary because additional drug would not have reached the affected muscles during that time. It will be important to establish whether treatment with dantrolene from the beginning of the reperfusion period alone influences the muscle damage, since this is the probable timing of any clinically relevant intervention.

The data presented therefore provide biochemical

evidence of a role for free radical species and/or an accumulation of intracellular calcium in the damage to skeletal muscle following ischemia and subsequent reperfusion. They also suggest that dantrolene sodium may have a role in preventing some aspects of reperfusion-induced damage to skeletal muscle, following prolonged limb ischemia.

Acknowledgements

The authors wish to thank the Restoration and Functional Therapy (RAFT) Trust and the Sir Jules Thorn Charitable Trust for financial support, Professor Richard Edwards and Dr Jonathan Gower for helpful discussions, Mr E J Parnell for providing information concerning the effects of temperature on the ischemic rabbit limb, together with data from his pilot studies.

References

Anderson M E. Determination of glutathione and glutathione disulphide. Methods Enzymol 1985; 113: 548–55.

Bertorini T, Palmieri G, Bhattacharya S. Beneficial effects of dantrolene sodium in exercise-induced muscle pains: Calcium mediated? Lancet 1982; i: 616–7.

DiMonte D, Rose D, Bellemo G, Eklow L, Orrenius S. Alterations in intracellular thiol homeostasis during the metabolism of menadione by isolated rat hepatocytes. Arch Biochem Biophys 1984; 235: 334–42.

Duncan C J, Smith J L, Greenway H C. Failure to protect frog skeletal muscle from ionophore-induced damage by the use of the protease inhibitor leupeptin. Comp Biochem Physiol 1979; 63C: 205-7.

Ellis K O, Carpenter J F. Studies of the mechanism of action of dantrolene sodium. A skeletal muscle relaxant. Naunyn Schmiedebergs Arch Pharmacol 1972; 273: 83–94.

Gronert G A. Malignant hyperthermia. Anaesthesiology 1980; 53: 35–63.

Halliwell B, Gutteridge J M C. Free radicals in medicine and biology. Clarendon Press, Oxford 1985.

Haverkort-Poels P J E, Joosten E M G, Ruitenbeek W. Prevention of recurrent exertional rhabdomyolysis by dantrolene sodium. Muscle Nerve 1987; 10: 45–6.

Jackson M J, Jones D A, Edwards R H T. Lipid peroxidation of skeletal muscle—an in vitro study. Biosci Rep 1983; 3: 609–19.

Jackson M J, Jones D A, Edwards R H T. Experimental skeletal muscle damage: The nature of the calcium-activated degenerative processes. Eur J Clin Invest 1984; 14: 369–74.

Jackson M J, Jones D A, Edwards R H T. Measurements of calcium and other elements in needle biopsy samples of muscle from patients with neuromuscular disorders. Clin Chim Acta 1985; 147: 215–21.

Jones D A, Jackson M J, McPhail G, Edwards R H T. Experimental muscle damage: The importance of external calcium. Clin Sci 1984; 66: 317–22.

Lindsay T F, Romaschin A D, Walker P M. Free radical mediated damage in skeletal muscle. Microcirc Endothelium Lymphatics 1989; 5: 157–70.

McArdle A, Edwards R H T, Jackson M J. Accumulation of calcium by normal and dystrophin-deficient muscle during contractile activity 'in vitro'. Clin Sci 1992; 82: 455–9.

McCord J M Oxygen-derived free radicals in post-ischemia tissue injury. N Engl J. Med 1985; 312: 159–61.

Morgan K G, Bryant S H. The mechanism of action of dantrolene sodium. J Pharmacol Exp Ther 1977; 201: 138–47.

Oredsson S, Plate G, Quarfordt P. Allopurinol—a free radical scavenger—reduces reperfusion injury in skeletal muscle. Eur J Vasc Surg 1991; 5: 47–52.

Pang C Y. Ischemia-induced reperfusion injury in muscle flaps: pathogenesis and major source of free radicals. J Reconstr Microsurg 1990; 6: 77–83.

Perler B A, Tohmeh A G, Bulkley G B. Inhibition of the compartment syndrome by the ablation of free radical-mediated reperfusion injury. Surgery 1990; 108: 40–7.

Rhechnagel R O, Ghoshall A K. Quantitative estimation of peroxidative degeneration of rat liver microsomal and mitochondrial lipids after carbon tetrachloride poisoning. Exp Mol Pathol 1966; 5: 413–26.

Walker P M. Ischaemia/reperfusion injury in skeletal muscle. Ann Vasc Surg 1991; 5: 399–402.

Wrogemann K, Pena S D J. Mitochondrial calcium overload: a general mechanism for cell necrosis in muscle diseases. Lancet 1976; 1: 672–4.