Ischemia-reperfusion-induced muscle damage

Protective effect of corticosteroids and antioxidants in rabbits

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We examined the potential protective effect of pretreatment with corticosteroids or antioxidants (ascorbic acid or allopurinol) in rabbits with reperfusion-induced damage to skeletal muscle after ischemia.

4 hours of limb ischemia induced by a pneumatic tourniquet, followed by reperfusion for 1 hour, caused a considerable amount of ultrastructural damage to the anterior tibialis muscles accompanied by a rise in circulating creatine kinase activity. Pretreatment of animals with depomedrone by a single 8 mg bolus injection led to a preservation of the anterior tibialis structure on both light and electron microscopy. High-dose continuous intravenous infusion with ascorbic acid (80 mg/hr) throughout the period of ischemia and reperfusion also preserved skeletal muscle structure, although allopurinol in various doses had no protective effect.

These data are fully compatible with a mechanism of ischemia/reperfusion-induced injury to skeletal muscle, involving generation of oxygen radicals and neutrophil sequestration and activation. They also indicate that damage to human skeletal muscle caused by prolonged use of a tourniquet is likely to be reduced by simple pharmacological interventions.

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The mechanisms causing damage to muscle following ischemia and reperfusion have been the subject of a number of recent studies. These have indicated that oxygen radicals may play a role (Perler et al. 1990, Oredsson et al. 1991). The source of these radicals has not been clarified, although by analogy with data from other tissues, it is possible that generation of superoxide ions by xanthine oxidase activity (McCord 1985) and/or generation by neutrophils or macrophages sequestered in the reperfused tissue (Hickey et al. 1993, Suematsu et al. 1994) are the primary sources.

In order to evaluate the possibility that inhibition of these pathways may prevent reperfusion damage to skeletal muscle and protect the limb from ischemia, we have examined the effect of allopurinol (an inhibitor of xanthine oxidase activity), ascorbic acid (a water-soluble reducing agent which acts to scavenge free radicals) and a long-acting corticosteroid (which can inhibit neutrophil sequestration and activation) on a standardized model of limb ischemia in the rabbit.

Animals and methods

New Zealand white rabbits (weight approximately 3.5 kg) maintained on a standard laboratory feeding regimen were used for all studies. Each animal was 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.

Ischemia followed by reperfusion of the limb was induced using a pneumatic tourniquet, as previously described (Klenerman et al. 1995). 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 prior to killing. Muscle samples from the soleus, anterior tibialis and quadriceps (distal to the tourniquet) from both legs were removed and small strips fixed in 3% glutaraldehyde for microscopic analysis. The rest of the muscle samples were rapidly frozen in liquid nitrogen for chemical analysis.

Where administered, allopurinol was given by continuous i.v. infusion at a rate of 4.5, 9 or 12 mg/hr throughout the experiment, ascorbic acid was given

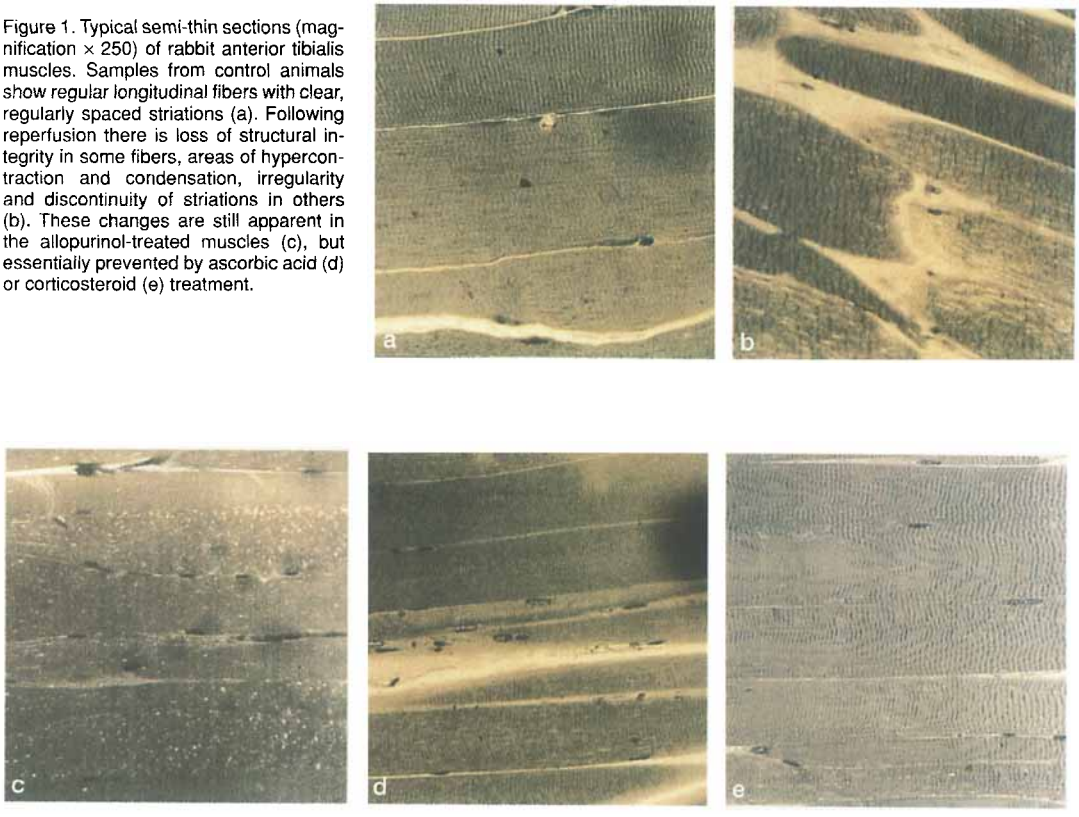

Figure 1. Typical semi-thin sections (magnification × 250) of rabbit anterior tibialis muscles. Samples from control animals show regular longitudinal fibers with clear, regularly spaced striations (a). Following reperfusion there is loss of structural integrity in some fibers, areas of hypercontraction and condensation, irregularity and discontinuity of striations in others (b). These changes are still apparent in the allopurinol-treated muscles (c), but essentially prevented by ascorbic acid (d) or corticosteroid (e) treatment.

by i.v. infusion at a rate of 80 mg/hr throughout the experiment or depomedrone was given by a single intramuscular injection (8 mg) 30 min prior to commencing the experiment.

Microscopic analysis

The fixed strips of muscle were post-fixed in buffered osmium tetroxide, dehydrated in alcohols of increasing strength and embedded in Epon Araldite (Agar Scientific). Semi-thin longitudinal sections (1.5–2.0 μm) were stained using 1% toluidine blue (Gurr, BDH Ltd.) in 1% borax buffer. The extent of damage to fibers was quantified from the semi-thin sections by point-counting, using a square lattice grid. At least 6 sections from each muscle were examined. Thin sections (90–120 nm) were cut for transmission electron microscopy and mounted on 200 mesh copper grids. Grids were stained with uranyl acetate and lead citrate and examined in a Philips CM-10 electron microscope.

Chemical analyses

The creatine kinase activity of serum samples obtained when the animals were killed, was analyzed as previously described (Jones et al. 1983). Muscle cation content was analyzed on freeze-dried samples of muscle, as previously described (Jackson et al. 1985).

The thiobarbituric acid reactivity (TBARS) and diene conjugate content of samples were analyzed as indicators of muscle-free radical activity, using methods previously described (Klenerman et al. 1995).

The muscle myeloperoxidase activity was measured as an index of the extent of neutrophil sequestration by the method described by Seekamp et al. (1993). All reagents used for these analyses were of Analar grade or the highest grade commercially available.

Statistics

Statistical analysis of results was performed with Student's t-test, except where data were non-gaussian in distribution, in which case the Mann-Whitney/Wilcoxon rank sum test was applied. A p-value of < 0.05 was considered significant.

Results

4 hours of limb ischemia, followed by 1 hour of reperfusion, caused changes in muscle structure similar to those reported previously (Bushell et al. 1995, Klenerman et al. 1995). These included mitochondria which were swollen, dilated sarcoplasmic reticulum,

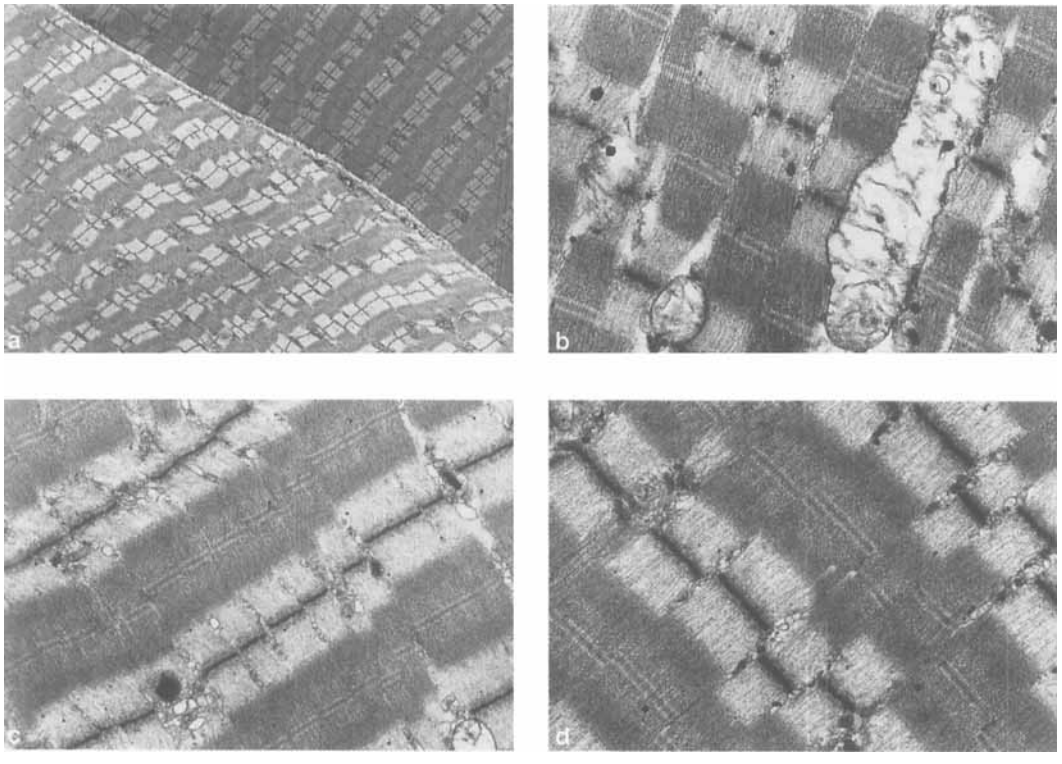

Figure 2. Typical ultrastructural appearance of rabbit anterior tibialis from control (a), reperfused (b) and reperfused limbs treated with ascorbic acid (c) or corticosteroids (d). Magnification: (a) × 2950, (b) × 15500, (c) × 11500 and (d) × 15500. Reperfusion caused loss of structural integrity with swollen and disrupted mitochondria. Some loss of myofibrillar and Z line structure (b). These changes were effectively prevented by ascorbic acid (c) or corticosteroids (d).

Table 1. Serum creatine kinase activity (U/L) of rabbits following 4 hours of unilateral limb ischemia and 1 hour of reperfusion. Mean *SEM*

	n	CK activity
Non-tourniquet-treated	10	201 *27*
Untreated reperfused animals	10	474 *54*
Allopurinol-treated (9 mg/hr)	6	504 *70*
Ascorbic acid-treated	5	302 *46**
Corticosteroid-treated	6	598 *37*

*Significantly reduced compared with untreated reperfused animals, $p < 0.05$.

some streaming of the Z lines and loss of glycogen on electron microscopic examination. These degenerative changes were most prominent in the tibialis anterior muscle, where point-counting revealed 79 (2)% (mean (SEM)) damaged sites on semi-thin sections (Figures 1 and 2). Muscle damage was also apparent in the serum creatine kinase activity (Table 1) and was associated with a rise in the muscle calcium and sodium content (Table 2), but no increase in the muscle content of the indicators of free radical activity was seen (Table 2). The damaged muscle also appeared to contain a higher number of neutrophils, since the myeloperoxidase activity was significantly elevated in the reperfused muscle (Table 3).

Effect of allopurinol

Allopurinol was administered to groups of 6 animals in 3 different doses (4.5, 9 or 12 mg/hr), but no protective effect against reperfusion-induced muscle damage was seen at any dose (Figure 1, Tables 1 and 2). In contrast, myeloperoxidase activity was not elevated in the reperfused muscle of the allopurinol-treated animals (Table 3).

Effect of ascorbic acid

High-dose treatment with ascorbic acid caused a definite preservation of muscle integrity following reperfusion (Figure 1). This protection was observed in all 5 animals studied and the number of damaged sites was significantly reduced to 6 (1)%. The protective effect was also observed at the ultrastructural level (Figure 2) and was apparent as a significant reduction in serum creatine kinase activity (Table 1). A signifi-

Table 2. Muscle calcium, sodium, TBARS and diene conjugate contents of anterior tibialis muscles following 4 hours of ischemia and 1 hour of reperfusion. Mean *SEM*

	Calcium (μmol/g dry wt)		Sodium (μmol/g dry wt)		TBARS		Diene conjugates	
Non-tourniquet-treated, n 10	3.5	*1.6*	129	*23*	–		–	
Untreated reperfused muscles, n 10	5.3	*1.2*	265	*35*	1.1	*0.29*	1.0	*0.2*
Allopurinol-treated, reperfused(9mg/hr), n 6	7.2	*0.7*	222	*23*	1.25	*0.2*	2.0	*0.8*
Ascorbic acid-treated, reperfused, n 5	16.7	*6.9*	196	*9.6**	1.08	*0.11*	1.5	*0.4*
Corticosteroid-treated, reperfused, n 6	16	*3.3*	300	*28*	1.0	*0.1*	2.2	*1.1*

TBARS and diene conjugates were measured as previously (Klenerman et al. 1995) but are presented as the ratio of the content in the reperfused to the contralateral control limb to reduce the variability due to effects of treatment on the control contralateral muscle between animals.
*Value significantly reduced compared with untreated reperfused muscles, $p < 0.05$.

Table 3. Muscle myeloperoxidase activity (change in absorbance/min/g muscle). Mean *SEM* of data from anterior tibialis muscles. Numbers of animals are presented in brackets

Treatment	Control leg		Reperfused leg	
None (10)	0.17	*0.02*	0.58	*0.20**
Allopurinol (9mg/hr) (6)	0.15	*0.02*	0.31	*0.03*
Ascorbic acid (5)	0.12	*0.03*	0.23	*0.04*
Corticosteroid (6)	0.16	*0.01*	0.29	*0.02*

*Value significantly greater than control leg, $p < 0.05$.

cant reduction in muscle sodium content was also seen, but muscle calcium content remained elevated, no differences in indicators of free radical activity were seen and the myeloperoxidase activity of the reperfused muscle was not elevated in these animals (Tables 2 and 3).

Effect of corticosteroid treatment

Depomedrone treatment of 6 animals also reduced the disruption of muscle structure caused by reperfusion (Figures 1 and 2). Point-counting indicated that the number of damaged sites on semi-thin sections was reduced to 10 (2)%. No reduction in serum creatine kinase activity and no significant effects on muscle cation content or indicators of free radical activity were seen (Tables 1 and 2). Myeloperoxidase activity was not elevated in the reperfused muscles of treated animals.

Discussion

Our previous findings have indicated that a period of ischemia followed by reperfusion has a greater effect on the tibialis anterior muscle of rabbits than on the soleus or quadriceps muscle (Klenerman et al. 1995). This appears to be due to the greater number of type II fibers in the tibialis anterior than in the other muscles (Bushell et al. 1995). Damage to these muscles can be rapidly and conveniently monitored by examining semi-thin sections by light microscopy (Figure 1; Bushell et al. 1995). Our previous data have also indicated an increase in muscle free radical activity and/or a failure of muscle calcium homeostasis in the mechanisms of this damage and indicated that treatment with dantrolene sodium (an inhibitor of calcium release from the sarcoplasmic reticulum) provided protection against ischemia/reperfusion-induced muscle damage (Klenerman et al. 1995). The data presented in Table 2 do not support our previous findings of an increase in the non-specific indicators of free radical activity (TBARS and diene conjugates) in the reperfused tibialis anterior muscle (Klenerman et al. 1995). We cannot explain this discrepancy, since the experimental model was similar. However, both of these assays have been criticized for their lack of specificity and reliability (Halliwell and Gutteridge 1989, Grootveld and Rhodes 1995), which may explain the variability observed.

A considerable amount of previous data have implicated the generation of superoxide radicals by xanthine oxidase enzymes in the damage to ischemic tissue which occurs on reperfusion (e.g., see McCord, 1985). Allopurinol or its metabolite oxypurinol is an inhibitor of xanthine oxidase and the protective effects of this compound in some models of reperfusion-induced tissue injury have been attributed to this property of the drug. Some previous data support a role for allopurinol in protecting skeletal muscle against reperfusion-induced damage (Godin and Bhimji 1987, Oredsson et al. 1991), but the data reported here do not support this. The reason for this disagareement is unclear although considerable debate continues concerning the possible role of xanthine oxidase in reperfusion injury (Richard et al. 1993) and whether xanthine oxidase is present in skeletal muscle (Hellsten-Westing 1993).

Ascorbate proved to be very effective in preserving

the structure of the reperfused anterior tibialis muscle. A lipid soluble preparation of ascorbic acid (2-0-octadecylascorbic acid) has proved to have some beneficial effects against post-ischemic reperfusion injury to rat skeletal muscle (Kondou et al. 1993), although these authors reported beneficial effects only on muscle ATP content and the lipid peroxides found in serum and did not examine muscle structure. The beneficial effects on muscle structure which we observed were not associated with biochemical evidence of a reduction in free radical activity (Table 2).

Many recent reports have indicated an accumulation of neutrophils within the microvasculature in the injury to tissues which follows reperfusion (e.g., see Chen et al. 1994). Corticosteroids can reduce both the activation and sequestration of neutrophils in inflamed tissue and this may be the basis of their beneficial effect in reperfused muscle tissue. We utilized a long-acting steroid which was administered 30 min prior to the experiment (i.e., 4 1/2 hours prior to reperfusion). Steroids are thought to reduce the production of neutrophil activators and chemoattractants, such as thromboxane, by activating synthesis of regulatory proteins, including the lipocortins (Browning et al. 1990). These substances should probably be administered for a sufficient period before reperfusion to allow this synthesis to occur.

Myeloperoxidase activity in tissues has been shown to correlate directly with the number of neutrophils present (Allan et al. 1985) and the data reported here indicate that the neutrophil content of the reperfused muscles would be increased (Table 3). However, such an increase was prevented by all of the compounds tested. The implication of this appears to be that the extent of neutrophil sequestration is not directly related to the structural damage seen.

In summary, the pharmacological data reported here support the general concept that reperfusion injury to skeletal muscle is caused by an initial production of oxygen radicals on reperfusion and that this leads to endothelial dysfunction, with neutrophil sequestration and consequent release of further oxygen radicals, elastase etc. However, our data provide no support for the possibility that xanthine oxidase is the initial source of oxygen radicals. The relatively simple pharmacological interventions which gave protection against experimental reperfusion injury to rabbit muscle in this study may prove useful in clinical practice. Thus it should be possible to protect the limb with a bloodless field and a tourniquet from some of the morbidity associated with prolonged (i.e., 1 1/2 to 2 hours) use of the tourniquet. A randomized clinical trial of potential protective agents now seems warranted. A further clinical application of this work may be the pharmacological protection of a limb prior to release of an impending compartment syndrome.

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