Intracellular calcium, cell injury and relationships to free radicals and fatty acid metabolism

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An accumulation of calcium in intracellular sites has been implicated in the mechanisms of cellular damage in various cell types. In cardiac tissue, damage due to hypoxia or re-oxygenation has been shown to be associated with an increase in tissue Ca content (Nayler *et al.* 1979), and more recently rises in the free intracellular Ca content have been described in isolated myocytes subjected to 'chemically-induced hypoxia' (Allshire *et al.* 1987). In hepatocytes loss of cell viability following incubation with various toxins was reported to be greatly reduced when the external Ca was removed from the incubation fluid (Schanne *et al.* 1979), suggesting a general requirement for external Ca in the damage process, but this finding has been the subject of much controversy (e.g. Smith *et al.* 1981; Farris *et al.* 1985). The general hypothesis that cell injury or death induced by various factors is mediated by a rise in the free intracellular Ca content is an attractive one because of the large difference in intracellular and extracellular Ca concentrations. Thus, a minor change in the plasma membrane could modify the permeability to Ca sufficient to allow a relatively large amount of Ca to enter the cell down the large concentration gradient for the element.

We have studied the mechanisms underlying the loss of cell activity in skeletal muscle. Much of this work has been undertaken using an in vitro skeletal muscle incubation system (Jones *et al.* 1983) in which the muscle can be damaged by excessive contractile activity, administration of metabolic poisons, detergents or toxins. Damage to the muscle fibres can be assessed by measuring the efflux of cytosolic enzymes (Jones *et al.* 1983), histological examination (Jones *et al.* 1984) or examination of the ultrastructure using electron-microscopic techniques (Duncan & Jackson, 1987). It is important for the study of the processes of damage that the tissue to be studied is undamaged during isolation, but because of the physical nature of skeletal muscle cells, dissection of untraumatized intact muscles is difficult, and in practise only one or two pennate muscles from rodents are suitable. Most work has been undertaken with mouse or rat soleus muscles, although the extensor digitorum longus is also entirely suitable. Strips of human skeletal muscle have also been examined in such systems (Anand & Emery, 1980; Jackson *et al.* 1990), although the flux of cytosolic enzymes from these preparations is considerably greater than from intact animal muscle and the validity of their use has yet to be proven.

Dependence of skeletal muscle damage on Ca

High external Ca concentrations (3–10 mmol/l) have been shown to increase cytosolic creatine kinase (*EC* 2.7.3.2; CK) release from isolated animal (Soybell *et al.* 1978) and human skeletal muscle (Anand & Emery, 1980). Treatment of skeletal muscle preparations with the Ca ionophore, A23187, has also demonstrated the potential of increased Ca levels to induce damage (Duncan *et al.* 1979). Slow Ca-channel blocking

agents (i.e. Ca antagonists) have been found to reduce CK release from human skeletal muscle in vitro (Anand & Emery, 1982), and other workers have shown that alternative manipulations designed to reduce Ca accumulation in skeletal muscle (i.e. parathyroidectomy) prevents much of the pathological changes to skeletal muscle in hamsters with congenital forms of muscular dystrophy (Palmieri *et al.* 1981). In another pathological condition in muscle (selenium-deficiency myopathy) ^{45}Ca accumulation by muscles has been reported to precede biochemical, histological or clinical evidence of myopathy (Godwin *et al.* 1975), and in the mdx mouse model of muscular dystrophy muscle free-intracellular Ca levels are elevated (Turner *et al.* 1988).

Studies using the previously described in vitro skeletal muscle damage system have indicated that the release of cytosolic enzymes following various different stresses (excess contractile activity, treatment with low-dose detergents or with mitochondrial inhibitors) could be greatly reduced by removal of the external Ca during the damaging period (Jackson *et al.* 1984; Jones *et al.* 1984). It was also found that this manipulation was effective in protection of the muscle against the histological and ultrastructural changes induced by excess contractile activity (Jones *et al.* 1984), although later work suggested the effects of the mitochondrial inhibitor 2,4-dinitrophenol on muscle ultrastructure were not reduced in the absence of extracellular Ca (Duncan & Jackson, 1987). Other studies have demonstrated a dramatic increase in total muscle Ca following either excess contractile activity leading to enzyme efflux, or treatment with mitochondrial inhibitors (Claremont *et al.* 1984).

These results suggest that the processes underlying cytosolic enzyme efflux from damaged skeletal muscle, and those responsible for some of the ultrastructural changes, are mediated by a net influx of extracellular Ca down the large extracellular–intracellular concentration gradient for this element. The increased intracellular Ca content then appears to activate further degenerative pathways.

The nature of these further Ca-activated degenerative pathways has been the subject of much of our recent work. Ca-induced membrane phospholipid hydrolysis via activation of phospholipase enzymes appears to be the key step in the mechanisms leading to efflux of intracellular enzymes (Jackson *et al.* 1984), although Ca-induced mitochondrial damage also occurs (Wrogemann & Pena, 1976), but its relevance to acute damage to the muscle cell is uncertain.

Relationship of Ca-induced damage to membrane fatty acid metabolism

It has been previously stated that in skeletal muscle a key step in the process leading to the loss of cell viability and efflux of cytosolic enzymes is activation of phospholipase enzymes. This leads to a release of prostaglandins from the muscles in association with the release of CK and other cytosolic enzymes (Jackson *et al.* 1987). Pharmacological agents having inhibitory effects on cyclo-oxygenase enzymes catalysing prostaglandin formation were found to prevent the efflux, but had no effect on CK release (Jackson *et al.* 1987). Cyclo-oxygenase products cannot, therefore, be direct mediators of the loss of cell viability which occurs during experimental skeletal muscle damage in vitro.

It therefore appears that the Ca accumulation seen during damage to skeletal muscle induces release of arachidonic acid from membrane phospholipids by the activation of phospholipase A_2 (*EC* 3.1.1.4; although phospholipase C (*EC* 3.1.4.3) may also be involved in this process). It is apparent that the released arachidonic acid provides the

substrate for prostaglandin production, but in many tissues other highly active metabolites (such as leukotrienes or hydroxyeicosatetraenoic acids) are produced from arachidonic acid via lipoxygenase (or lipoxidase; *EC* 1.13.11.12) activity. Lipoxygenase enzymes have not been described or isolated from skeletal muscle tissue, but putative inhibitors of these enzymes were found to significantly reduce the cytosolic enzyme efflux from tissues treated with either 2,4-dinitrophenol or the Ca ionophore, A23187 (Jackson *et al.* 1987). However, despite a considerable amount of work we have been unable to demonstrate the existence of lipoxygenase activity or the production of lipoxygenase products from skeletal muscle, and the presence of these enzymes in this tissue must remain open to question.

Since it appeared likely that a product or products of phospholipase A_2 activity was responsible for the loss of viability of muscle cells following intracellular Ca overload, we have attempted to modify skeletal muscle fatty acid composition in order to alter the products of phospholipase A_2 activation. Diets rich in long-chain *n*-3 fatty acids have been extensively used to modify membrane composition and the products of phospholipase activation, and giving diets rich in marine oils to rats caused substantial replacement of the muscle membrane arachidonic acid (20:4*n*-6) with eicosapentaenoic acid (20:5*n*-3) and docosahexaenoic acid (22:6*n*-3) (Jackson *et al.* 1988). If specific metabolites of arachidonic acid were responsible for the changes in membrane permeability to large proteins associated with damage, this nutritional manipulation would be expected to have protective effects, but muscles from the marine-oil-treated group of rats showed increased susceptibility to the effects of Ca ionophore-induced intracellular Ca overload (Jackson *et al.* 1988).

Possible explanations for these findings are that lipoxygenase enzymes, which have been hypothesized to be present in skeletal muscle (Jackson *et al.* 1987), can act on a wide spectrum of unsaturated fatty acids to produce damaging metabolites, or that some other process (such as non-enzymic lipid peroxidation) is activated during the damaging process and is influenced by the nature of the fatty acids present in the skeletal muscle membranes.

Relationship of Ca-induced damage to free radical metabolism

An increase in free radical-mediated reactions leading to muscle damage during exercise has been proposed by a number of workers (Dillard *et al.* 1978; Brady *et al.* 1979; Gee & Tappel, 1981; Davies *et al.* 1982). The major problem associated with all studies of free radical activity in tissues is the availability of suitable techniques. Most of the techniques utilized rely on non-specific, indirect indicators of free radical activity (see Jackson, 1987).

In heart muscle it has been proposed that increased free radical activity can lead to a failure of Ca homeostasis with consequent damage to the myocytes (Ferrari *et al.* 1986), whereas our findings initially suggested that during skeletal muscle damage the reverse may occur, i.e. Ca overload leads to an activation of free-radical-mediated processes.

Our initial findings, examining the effects of variation in the muscle vitamin E content on experimental damage to skeletal muscle, demonstrated an apparent protective effect of this vitamin (Jackson *et al.* 1983). Since the major role of vitamin E appears to be as a lipid-soluble antioxidant inhibiting free-radical-mediated lipid peroxidation, this finding suggested that free-radical-mediated damaging processes may be activated in this system.

Studies using electron-spin-resonance spectrometry appear to support this suggestion (Jackson *et al.* 1985; Johnson *et al.* 1988); however, recent findings obtained by examining the effects of extracellular α-tocopherol on normal muscle confirm the protective effects of vitamin E against skeletal muscle damage, but also suggests that it may be acting via a non-antioxidant mechanism (Phoenix *et al.* 1989).

In recent attempts to clarify the situation we have examined glutathione content and release from experimentally-damaged muscles. Glutathione is the major cellular reductant and one of the major 'protective' materials against oxidizing free radicals. It was found to be rapidly lost from skeletal muscle in response to excessive contractile activity or treatment with metabolic inhibitors, although this process did not appear to be crucially dependent on extracellular Ca or to be significantly stimulated by treatment of the muscles with the Ca ionophore (Jackson *et al.* 1990). This work does not, therefore, support the idea of activation of free-radical-mediated damaging processes by intracellular Ca overload, although such processes may be activated independently in skeletal muscle following excessive contractile activity or treatment with metabolic poisons.

Potential for nutritional modification of muscle to prevent or reduce Ca-induced skeletal muscle damage

The exacerbation of exercise-induced damage to skeletal muscle by vitamin E deficiency, the apparent protective effects of supra-normal extracellular vitamin E concentrations to normal muscle and the effects of modification of muscle fatty acid composition on muscle damage suggest that nutritional modification of skeletal muscle to prevent or reduce Ca-induced skeletal muscle damage may be possible. Currently available information suggests that this should be aimed at reduction of the unsaturation of skeletal muscle membranes or enhancement of the antioxidant status of muscles, or both. Such manipulation will require examination in appropriate animal models before testing in man, but may potentially offer benefit to patients with various acute degenerative skeletal muscle disorders, and also possibly to reduce the damaging effects of severe or unaccustomed exercise in normal subjects.

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