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Autophagic response to strenuous exercise in mouse skeletal muscle fibers

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Summary. Strenuous physical exercise induces necrosis of skeletal muscle fibers and increases lysosomal enzyme activities in surviving muscle fibers. This study examines the ultrastructural basis of the stimulation of the lysosomal system in mouse vastus medialis muscle during the appearance and repair of exercise-induced (9 h of running) injuries. Necrotic fibers appeared the day after exercise and an inflammatory response with the replacement of necrotic fibers by phagocytes was highest 2–3 days after exertion. Ultrastructural study of surviving muscle fibers revealed numerous autophagic vacuoles, residual bodies, and spheromembranous structures at the periphery of myofibers, especially in fibers adjacent to necrotic fibers. The autophagic response was most prominent between 2 and 7 days after exertion. Autophagic vacuoles with double or single limiting membranes contained mitochondria at various stages of degradation. Vacuolar and multilamellar structures were also observed in regenerating muscle fibers. The structure of injured skeletal muscle fibers returned to normal within 2 weeks. It is proposed that increased autophagic activity could be related to the breakdown of cellular constituents of surviving muscle fibers to provide structural elements for regenerating muscle fibers.

Key words: Autophagy – Muscles – Exertion

Introduction

Strenuous physical exercise depletes the store of energy-yielding substrates and induces certain physicochemical and morphologic changes in skeletal muscles (Simonson 1971). Transient edema and swelling of mitochondria have been observed in the muscles of exhausted rats (Gollnick et al. 1971). The swelling of mitochondria is, however, a fixation artefact and reflects increased responsiveness to osmotic stress (Gale 1981). Later, 2–3 days after

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heavy exercise, focal necrotic lesions together with inflammation appear in the muscles of exercised animals (Highman and Altland 1963; Schumann 1972; Hecht et al. 1975; Vihko et al. 1978a). Exercise-induced fiber injuries occur mainly in muscles containing a large number of red oxidative muscle fibers, and the regeneration of injured fibers occurs later.

The activities of lysosomal acid hydrolases increase in the injured skeletal muscles of exercised mice (Vihko et al. 1978b; Salminen and Vihko 1980). A histochemical study showed that the increase in enzyme activities was due to invading phagocytes rich in acid hydrolases and to the increase of lysosomal enzyme activities in surviving muscle fibers (Vihko et al. 1978a). Similar responses in lysosomal enzyme activities have been observed in necrotic myopathies caused by ischemia (Shannon et al. 1974) and dimethyl-para-phenylene diamine (Meijer and Israel 1979). The present study was undertaken to examine the ultrastructural basis of the stimulation of the lysosomal system in surviving muscle fibers during the appearance and repair of necrotic lesions induced by strenuous exercise.

Materials and methods

Animals and physical exertion. Male NMRI-mice, 5-months-old, were made to run at a speed of 13.5 m/min on a motor-driven treadmill with 6° uphill tracks for 9 h with two 10–15 min pauses after 3 h and 6 h of running. All the mice were able to perform this program. After the exertion the mice lived for 1 hour ($n=5$), or for 1 ($n=6$), 2 ($n=6$), 3 ($n=6$), 5 ($n=6$), 7 ($n=5$), 10 ($n=5$), and 15 ($n=6$) days before killing. The mice were housed under standard cage conditions with free access to solid food pellets (R_3, Astra Ewos, Sweden) and tap water. Temperature (20–22° C) and daylight rhythm (12 h light/12 h dark) were kept constant. The control mice ($n=7$) weighed 33.9 ± 0.6 g (S.E.). The only statistically significant deviation from the control value was the weight of the 1 h group, 29.3 ± 0.7 g ($p<0.001$).

Microscopy. Mice were killed by cervical dislocation. The deep red proximal part of vastus medialis was excised and cut into small pieces (approx. 1 mm^3), which were fixed by immersion in a formaldehyde (4%) and glutaraldehyde (1%) combination in phosphate buffer (4CF-1G) as recommended by McDowell and Trump (1976) and stored at 4° C for 3–5 days. Prolonged storage over 12 months in 4CF-1G does not affect the ultrastructure (McDowell and Trump 1976). The samples were post-fixed with 1% osmium tetroxide in s-collidine buffer for 1 h at 0–4° C, stained with 0.5% uranyl acetate, dehydrated and embedded in Epon. Thin sections from all exercised and control samples were cut with diamond knives on a Sorval Porter-Blum ultramicrotomy. The sections were stained with lead citrate and uranyl acetate and examined with a JEM 100 U electron microscope. Sections from some blocks were also cut for toluidine blue-staining and examined by light microscopy to show necrotic and inflammatory changes (Vihko et al. 1978a).

Enzyme assay. The corresponding part of vastus medialis to that used for electron microscopy was excised from the contralateral leg and the activity of β-glucuronidase (EC 3.2.1.31) was assayed as described earlier (Salminen and Vihko 1982). β-Glucuronidase activity was measured in order to provide an enzymatic characterization of lysosomal changes after exertion in whole muscle samples (Vihko et al. 1978b). Means and standard errors (S.E.) were calculated. Statistical analysis was performed using the Student's *t*-test.

Results

One h after strenuous exercise, edema was observed in some muscle fibers. Mitochondria and myofibrils were normal and autophagic vacuoles were

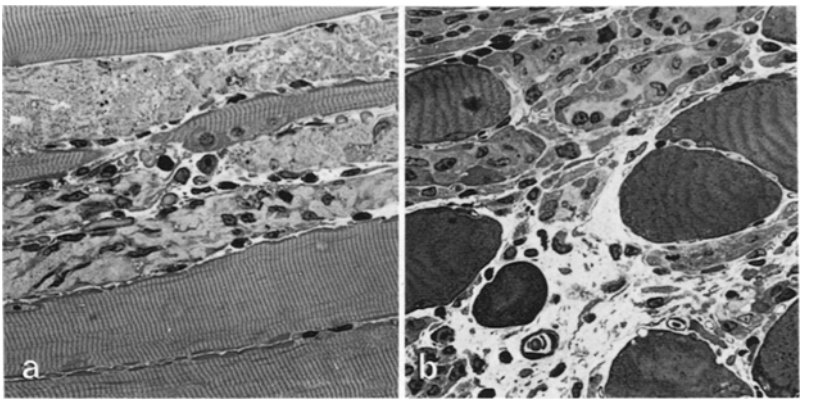

Fig. 1a, b. Toluidine blue-stained sections of mouse skeletal muscle after exertion. **a** A longitudinal section showing three necrotic muscle fibers and invasion of some inflammatory phagocytes (2 days after exertion). Magnification: × 213 **b** A transverse section showing necrotic muscle fibers replaced by clusters of mononuclear phagocytes (3 days after exertion). Magnification: × 340

infrequent or absent, as in the sedentary mice. Twenty-four h after exertion the edema had disappeared. However, some necrotizing fibers with segmental loss of Z-discs and disarray of myofilaments appeared. Some inflammatory cells were also present. Surviving muscle fibers sometimes displayed giant mitochondria and paracrystalline inclusions of peripheral mitochondria. Autophagic vacuoles, multilamellar whirled membranes and osmiofilic bodies were more frequent than in the sedentary mice.

Necrotic fibers together with inflammatory reaction were frequent in the red part of vastus medialis 2–3 days after strenuous exertion (Fig. 1). Invading phagocytes were observed inside necrotic fibers phagocytozing cell debris. Regenerative muscle fibers with abundant ribosomes and central nuclei appeared 5–7 days after exertion. The regeneration was well advanced 10 days after strenuous exercise.

The autophagic response after exertion was observed in surviving muscle fibers, especially in fibers adjacent to necrotic fibers. This response appeared during the period 2–7 days after exertion and was more prominent in fibers rich in mitochondria. Some autophagic vacuoles are presented in Fig. 2. Autophagic vacuoles were located mainly in the subsarcolemmal space but some vacuoles were also observed between the myofibrils (Fig. 2E). Autophagic vacuoles with double or single limiting membranes contained mitochondria, other membranous structures, or sarcoplasmic constituents such as glycogen granules. Mitochondria in autophagic vacuoles were at various stages of degradation (Fig. 2C, E). Sometimes segregated material was observed without a limiting membrane (Fig. 2A), or with the membrane broken up (Fig. 2C): this is probably a fixation artefact. Simultaneously with the occurrence of autophagic vacuoles an increased number of electrondense pleomorphic lipofuscin granules was observed in the subsarcolemmal area. Golgi complexes and multivesicular bodies appeared but lysosome-like structures were infrequent (Fig. 3).

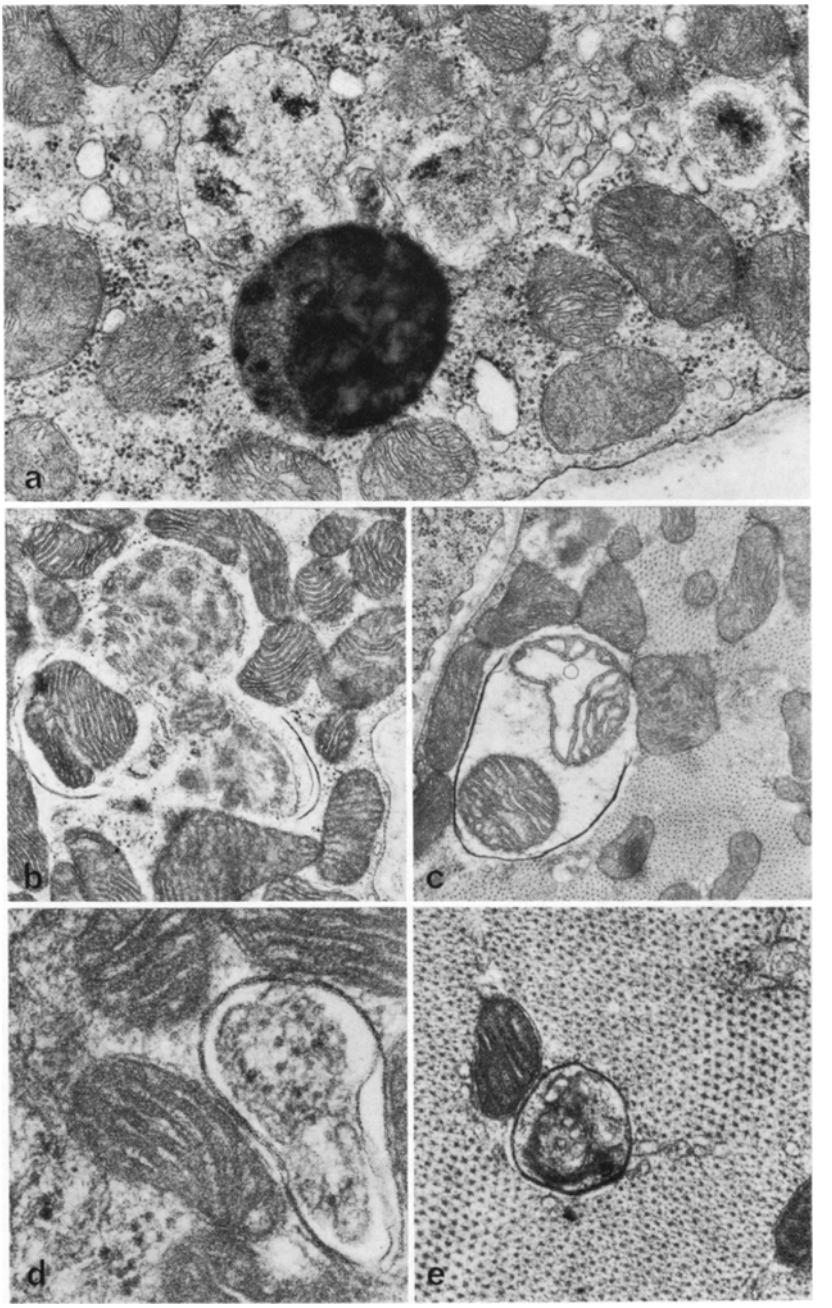

Fig. 2a–e. Electron micrographs of autophagic vacuoles in surviving muscle fibers after exertion. **a** Autophagic vacuoles, containing an unidentified degradation product, osmiophilic body, and Golgi complex, at the periphery of muscle fiber (5 days after exertion). Magnification: × 26,000 **b** Possibly a forming autophagic vacuole (3 days after exertion). Note decomposed mitochondria and cup-shaped cisternae. Magnification: × 19,500 **c** An autophagic vacuole containing partly degraded mitochondria (3 days after exertion). The membranes of autophagic vacuole are broken up, possibly due to the fixation. Magnification: × 19,500 **d** A small autophagic vacuole containing cytoplasmic material (3 days after exertion). Magnification: × 75,000 **e** A small autophagic vacuole between myofibrils (3 days after exertion). Magnification: × 40,000

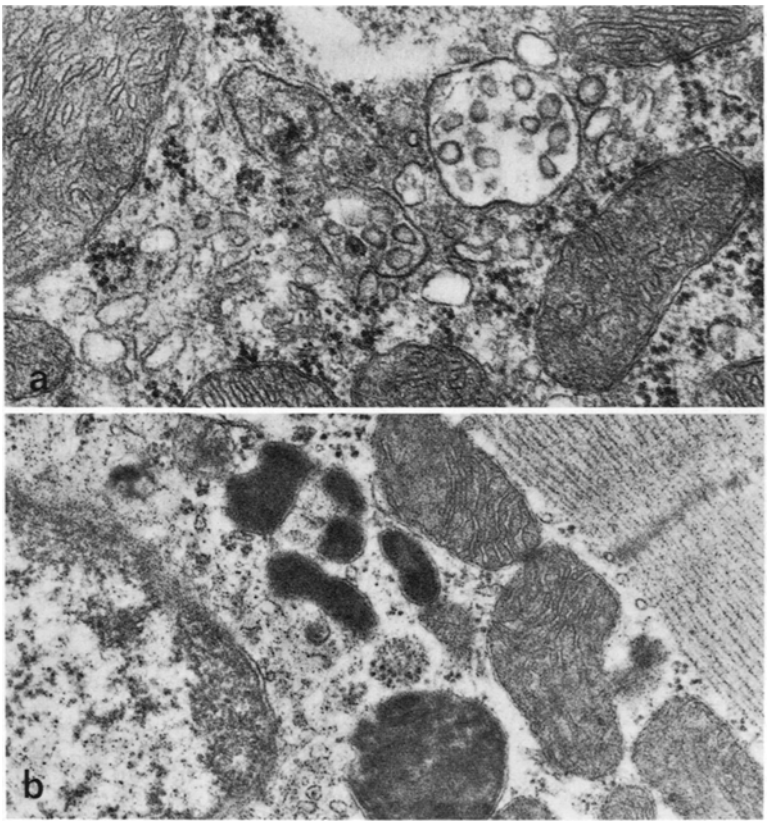

Fig. 3a. Multivesicular bodies (5 days after exertion). Magnification: × 40,000 **b** Membrane-bound dense granules (lysosome-like bodies) in the perinuclear region (3 days after exertion). Magnification: × 30,000

Another perceptible change in surviving muscle fibers was the accumulation of multilamellar whirled membranous structures, especially in the early phase of myopathy (Fig. 4). These spheromembranous bodies varied from small membranous whorls to large multilamellar myeloid bodies and occurred both beneath the sarcolemma and between the myofibrils. Multilamellar structures were also observed in the extracellular space, possibly due to exocytosis. Lamellar structures were infrequent in unexercised control mice.

Autophagic vacuoles, multilamellar bodies and osmiophilic structures were also frequently perceptible in regenerating muscle fibers. The structure of injured skeletal muscle returned to normal within 2 weeks, although some regenerated muscle fibers still contained central nuclei.

The activity of β-glucuronidase in vastus medialis markedly increased during the days following exertion (Fig. 5). The highest activity was observed on the 3rd post-exercise day. Thereafter the activity continuously decreased but was still twofold on the 15th day after exertion.

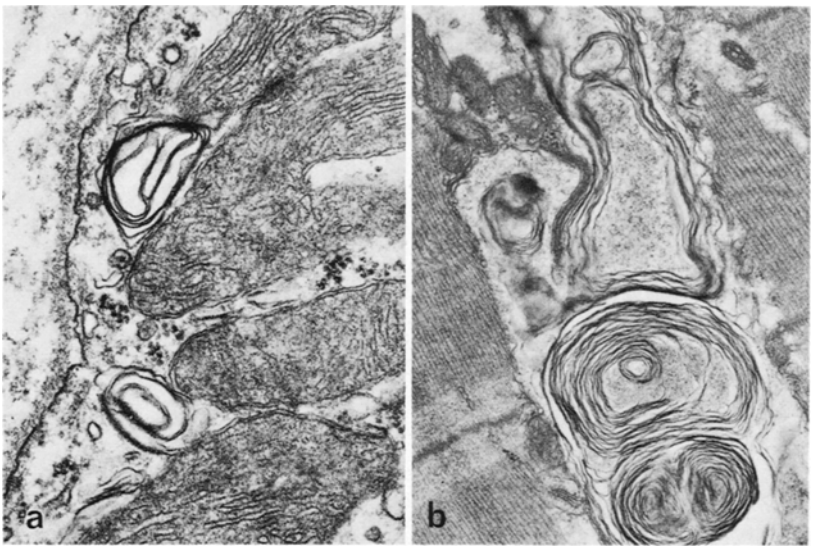

Fig. 4a, b. Multilamellar bodies of varying size in surviving muscle fibers after exertion. **a** Whirled lamellar bodies, deriving probably from the membranes of mitochondria (2 days after exertion). Magnification: × 40,500 **b** Large spheromembranous bodies between myofibrils (2 days after exertion). Magnification: × 19,500

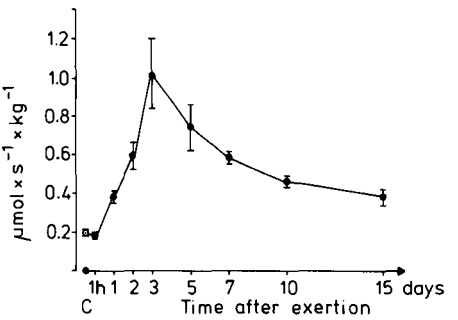

Fig. 5. The activity of β-glucuronidase in the red part of vastus medialis after exertion. Values are means ± S.E. All values of exercised groups, except that of the 1 h group, are significantly ($p < 0.001$) higher than the control value

Discussion

Strenuous exercise induced muscle fiber injuries in the red part of vastus medialis muscle. The first signs of incipient necrosis and inflammatory reaction were observed the day after strenuous exertion. Hence, our results are consistent with earlier studies suggesting that exercise myopathy is an ischemic compression syndrome appearing 1–2 days after exercise (Getzen and Carr 1967; Renemann 1968). Increased fluid accumulation in muscular tissues elevates the pressure, particularly in nonexpanding compartments, diminishes circulation and thus induces hypoxia and ischemia. The fiber injuries observed were of ischemic type and not mechanical injuries of myofibrillar structures as observed after eccentric exercise in man (Friden et al. 1981). Injured muscle fibers were repaired by a regeneration mechanism

similar to that described in connection with several types of muscle injuries (Reznik 1973).

The ultrastructural study revealed both auto- and heterophagocytosis in myopathic muscles. Invading phagocytes were removing the debris of necrotic muscle fibers by heterophagic uptake. On the other hand, an increased number of autophagic vacuoles was present in the surviving muscle fibers, particularly those in the area of necrotic lesions. Vacuoles were most probably of autophagic origin, because the endocytotic ability of muscle fibers is limited and the material in the autophagic vacuoles was decomposed to different degrees suggesting organelle breakdown. No indication of bulk endocytosis was observed, even in fibers bordering necrotic fibers. Conversely, some indications of the exocytosis of spheromembranous bodies were observed. The exocytosis of autophagic vacuoles and spheromembranous bodies has been described in chloroquine myopathy (MacDonald and Engel 1970; Schmalbruch 1980).

Increased cellular autophagy is observed as a response to many pathologic influences and to the deprivation of essential nutrients or in the course of differentiation, metamorphosis, and involution (Ericsson 1973; Kerr 1973; Helminen 1975). Decker and Wildenthal (1980) described a considerable increase in the number of autophagic vacuoles in heart muscle during reoxygenation after hypoxic perfusion. They suggested that lysosomal autophagy is an important repair mechanism of hypoxia-induced sublethal injuries. Autophagic vacuoles have also been found in human skeletal muscle after temporary incomplete ischemia (Sjöström et al. 1982) and during intermittent claudication (Sjöström et al. 1980). In addition to hypoxia, chloroquine (MacDonald and Engel 1970; Schmalbruch 1980) and vincristine (Clarke et al. 1972) administration, denervation (Schiaffino and Hanzlikova 1972) or vitamin E deficiency (Lin and Chen 1982), may stimulate autophagy in skeletal muscle fibers.

The inducing factors for autophagic uptake in association with exercise injuries are far from clear. Strenuous exercise as such does not stimulate autophagic uptake, because both autophagic vacuoles and spheromembranous bodies were infrequent 1 h after exercise. During the 1–2 post-exercise days hypoxia and hypoxic injuries could be the stimulating agent, as suggested by Decker and Wildenthal (1980) in the case of the hypoxic myocardium. However, autophagic vacuoles and multilamellar bodies are more frequent 3–7 days after exertion in surviving muscle fibers.

Hence, it is unlikely that autophagic response reflects an attempt by the sarcoplasma to wall-off injured structures. Autophagic activity in fibers adjacent to regenerating and growing muscle fibers could be related to the breakdown of cellular constituents of surviving muscle fibers to provide the structural elements of growing muscle fibers. Changes in protein degradation are related in many tissues and conditions to changes in the volume of autophagic vacuoles (Pfeifer 1982). Hence, increased autophagic activity in surviving fibers during the regeneration phase would reflect augmented degradation and turnover. The increased number of multilamellar bodies might also be due to the increased turnover of membranous structures,

although spheromembranous bodies are usually associated with the inhibition of phospholipid degradation (Clarke et al. 1972).

Studies on the chloroquine-induced autophagic response have shown that the limiting membranes of autophagic vacuoles originate from t-tubules and not from the sarcoplasmic reticulum or Golgi complex (MacDonald and Engel 1970; Schmalbruch 1980). Cup-shaped and winding cisternae of t-tubules were also observed in the present study, probably being membranes of developing autophagic vacuoles. The segregation may also be to some extent selective in muscle fibers, as indicated by the morphometric approach in rat liver (Pfeifer 1978). For instance, contractile structures have not been observed in autophagic vacuoles, but in the case of mitochondria segregation is probably a random process. Little is known about the process of autophagic degradation in muscle fibers. For example, the role of primary and/or secondary lysosomes in the acquisition of hydrolytic enzymes by autophagic vacuoles is unclear. In the present study lysosome-like bodies were observed only infrequently and no fusions with autophagic vacuoles were found. Christie and Stoward (1977) have suggested that hydrolytic enzymes are transferred by the sarcoplasmic reticulum to developing autophagic vacuoles. However, segregated constituents have been decomposed in autophagic vacuoles and not removed immediately by exocytosis.

Our studies on lysosomal enzyme activities during exercise myopathy have demonstrated increased activities of acid hydrolases in myopathic muscles (Vihko et al. 1978a, b; Salminen and Vihko 1980). The present results are consistent with our earlier observations. Some of the enzyme activities originate in invading phagocytes, particularly in the early phase of the myopathy (Vihko et al. 1978a). In surviving muscle fibers the most intense histochemical staining patterns are observed during the 3–7 days after exertion (Vihko et al. 1978a). Increased cellular autophagy is not related to increased total acid hydrolase activities in the hypoxic myocardium of rabbit (Decker et al. 1980) or in the liver of vinblastine treated mice (Hirsimäki et al. 1976), but to the increase in soluble activities. Increased acid hydrolase activities in muscle fibers after strenuous exertion originate most probably in autophagic vacuoles, residual bodies, and possibly in spheromembranous bodies. This distribution of acid hydrolases has been observed in several myopathies (MacDonald and Engel 1970; Schiaffino and Hanzlikova 1972; Christie and Stoward 1977).

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