

The Fate of Dystrophin and Some Signs of Apoptosis in the Skeletal Muscle Work-Overloaded in Extension

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Abstract

Rat muscles, soleus and extensor digitorum longus (EDL), were eccentrically electrostimulated at low frequency for 4-24h. The cryostat-sections of these muscles were examined by immunohistochemistry. Numerous muscle fibres were damaged and necrotic. Simultaneously, several preserved fibres were not stained, or stained only discontinuously, with the anti-dystrophin and anti-p-dystroglycan antibody. Apoptotic nuclei (TUNEL-positive) were occasionally noticed within the muscle fibres. Those changes appeared earlier in the EDL than in the soleus. Key words: apoptosis, dystrophin, eccentric contraction, electrostimulation, muscle damage.

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As documented in the last decade, dystrophin [1,7, 23] and the sarcolemmal dystrophin-associated glycoprotein complex (DAG) [8], are very important for the mechanical and osmotic stability of sarcolemma [14, 17, 18]. These proteins are part of the structures called "costamers", which connect the contractile apparatus with the extracellular matrix [1,9, 16, 19]. In the dystrophic muscles the genetic defect of dystrophin, or one of the components of the DAG complex, is usually accompanied by loss of the other proteins within the complex [5, 8]. In fact, the loss of the DAG proteins is probably responsible for the most serious injuries of the dystrophic muscle fibres [16].

In the muscle maintained in a shortened or in an extended position the contractile structure is reorganized to obtain the optimal lengths of sarcomeres for the muscle function [22]. This process is accelerated when the muscle works intensively [11, 20]. However, the work-overloaded muscle can get damaged, particularly when it is simultaneously overextended [6, 10, 13]. The reason for this phenomenon is not clear, neither are the ways by which the fibres get damaged. It is possible, that some apoptotic processes take place in such a muscle. Possibility of apoptosis in the mature striated muscle has recently been documented [2, 4].

It is still unknown whether at all, and how, the dystrophin and DAG complex get affected in the damaged fibres of a work-overloaded muscle. Nor is the meaning known yet of the dystrophin and the DAG complex for the muscle adaptation to different condition of function and length. However, it seems unquestionable that during the contractile structure reorganization, dystrophin and DAG complex connections with the extracellular matrix get also

changed. Thus, "costamers" are bound to get reorganized in such a muscle and their proteins are expected to be affected.

In the present work the distribution of dystrophin and p-dystroglycan (dystrophin-associated glycoprotein, component of the DAG complex) were examined by immunohistochemical methods in the rat leg muscles electrically stimulated in the extended position for 4 h, 6 h and 24 h. In parallel, an examination by the "TUNEL" method was performed, to explain if any apoptotic processes develop in such muscle.

Methods

Animals

Three-month-old female albino Wistar rats were used. The soleus and *extensor digitorum longus* (EDL) muscles were maintained in an extended position by immobilisation of the ankle joint in a plastic tube at an angle of 90 or 160°, respectively. Stimulating electrodes were implanted on the sciatic nerve under pentobarbital and ether anaesthesia some days before the experiment. The sciatic nerve was stimulated continuously for 4h, 6h and 24h by pulses of 0.3 ms duration and 20 Hz frequency. As controls served: soleus muscles from nonexperimental animals of the same population and soleus muscle stimulated without joint immobilisation. In each group 2-4 animals were used.

Immediately after decapitation each whole muscle was excised, maintaining its length carefully, measured and attached to a plastic rod and frozen in isopentane cooled in liquid nitrogen. Muscles were stored at - 70°C.

Dystrophin in the overloaded-extended muscle

Histological examination was performed on cryostat-sections stained with hematoxylin and eosin.

Immunohistochemistry

Study with immunofluorescence microscope was performed on the 8 μ m cryostat-sections of muscle as previously described [3]. The sections were preincubated with 5% goat serum in PBS (0.1 M Na-phosphate pH 7.4, 0.15 M NaCl) for 30 min at room temperature. Monoclonal anti-

body anti COOH terminus of dystrophin (1:300) and anti p-dystroglycan (1:50) used as primary antibody were purchased at Novocastra, Newcastle upon Tyne, UK. After incubation with the primary antibody for 1 h at room temperature the sections were washed in PBS and reacted with anti-mouse antibody conjugated with fluorescein for 30 min at room temperature. After washing in PBS, the sections were examined in a Zeiss Axioplan microscope.

In situ DNA nick end labeling (TUNEL)

Serial tissue cryostat-section were made, and collected on polylysine precoated slides. *In situ* nick end labeling of fragmented DNA was performed using terminal deoxynucleotidyl transferase (TdT) and fluorescein-conjugated nucleotides with the *In Situ* Cell Death Detection Kit, POD (Boehringer Mannheim) as described by the manufacturer's instruction. Negative control slides were prepared by substituting distilled water for TdT. Enzyme and continuing with the staining procedure as suggested by the manufacturer's instructions. Labelled myonuclei were eas-

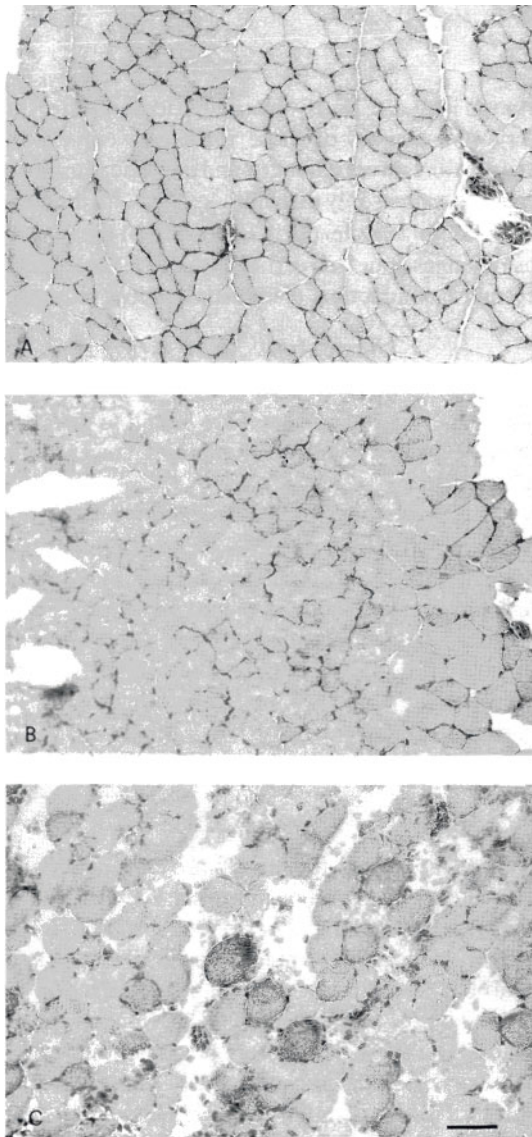


Figure 1. Cryostat-sections of the rat EDL muscle stained with hematoxylin and eosin. a) Control, b) 6 h stimulation in extension, c) 24 h stimulation in extension. Irregularity of the shape and dimension of fibres seen in b increased dramatically in c; damaged muscle fibres and infiltration by connective tissue cells seen in c. Bar = 57 μ m.

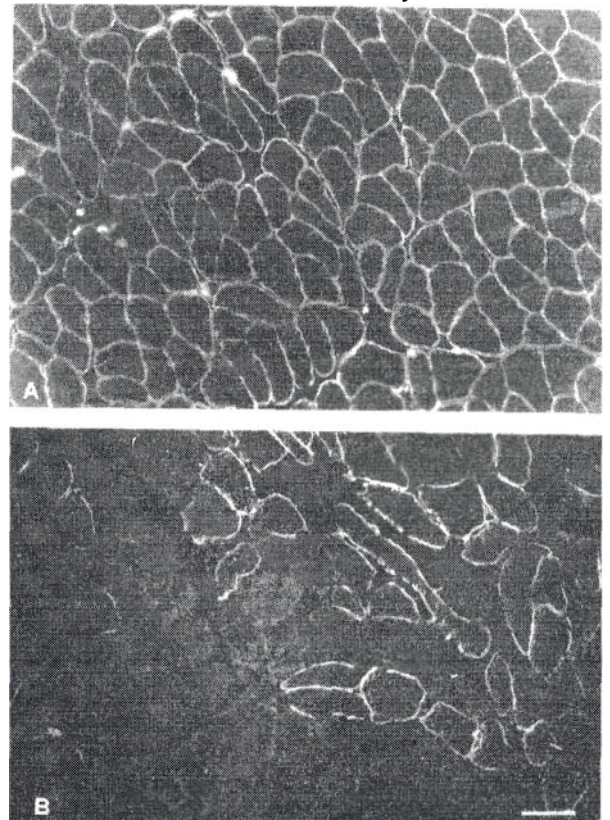
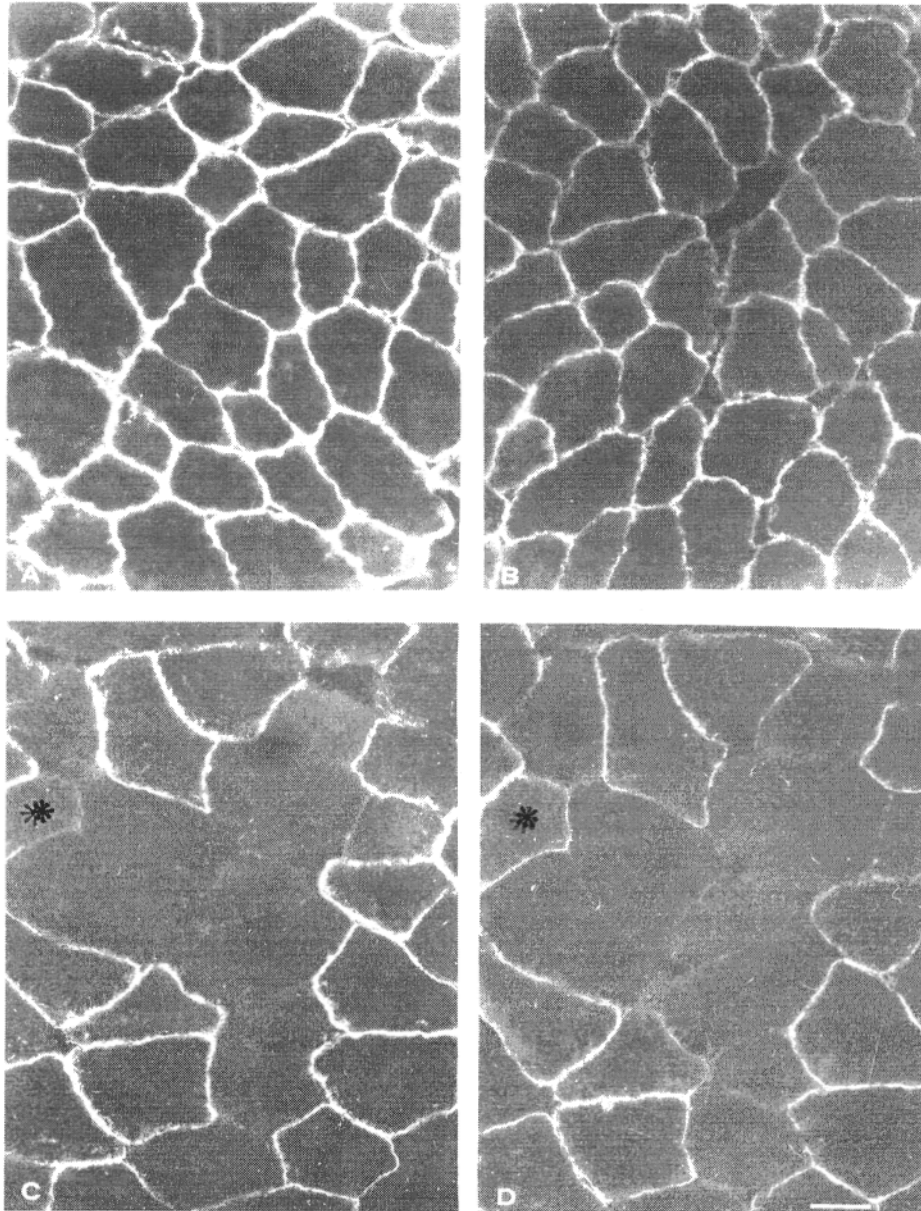


Figure 2. Soleus muscle immunolabelled with anti-dystrophin antibody, a) Control muscle. The immunolabel is continuously seen along the surface of the fibres, b) Muscle stimulated electrically in extension for 24 h. Signs of disarrangement of muscle fibres that become swollen and rounded or enlarged are seen in the left-hand side. Dystrophin is lacking in well-preserved fibres of normal shape and size - it is absent or else it is present discontinuously. Bar = 56 μ m.

Dystrophin in the overloaded-extended muscle

ily identified from the negative nuclei counter-stained by Hoechst 33258 and were photographed. Some sections were double-labelled for the presence of apoptotic nuclei and for laminin. Slides were incubated with primary antibody using the following dilution: rabbit anti-laminin (from Sigma Chemical Co., St. Louis, MO) diluted 1:10 in 1% BSA. The slides were then washed twice with PBS (5 min) and incubated with rhodamine-conjugated goat anti-

rabbit Ig (1:250 diluted in 1% BSA) for 1 h at 37°C. After the incubation with rhodamine conjugated antibody, the slides were rinsed in PBS, then were labelled for fragmented DNA according to the procedure described above for TdT nick-end labelling. After washing three times in PBS and after staining nuclei with Hoechst 33258, slides were mounted in Elvanol and observed under microscope.



^m Figure 3. EDL muscle immune/labelled with anti-dystrophin and anti-fi-dystroglycan antibody, control and stimulated electrically in extension for 4 h. a) Control muscle labelled with anti-dystrophin. b) Control muscle labelled with anti-fi-dystroglycan. c) Stimulated muscle, labelled with anti-dystrophin. d) Stimulated muscle, labelled with anti-fi-dystroglycan; c and d serial sections. In the control muscle the immunolabel is continuously seen along the surface of the fibres (a, b). In the stimulated muscle several fibres show normal shape and size, but lack of the dystrophin and fi-dystroglycan staining (the central parts of c and d); the fibre marked by asterisks is not reacting with anti-dystrophin antibody (c), whereas it is still labelled with anti-fi-dystroglycan antibody (d). Bar = 28 μ m.

Results

The control muscles showed regular pattern and shape of fibres, seen in the cryostat-sections both stained with hematoxylin-eosin (Fig. 1 a) and immunolabelled (Fig. 2a, 3a, b). The control soleus (Fig. 2a) and the control EDL (Fig. 3a) immunolabelled with anti-dystrophin showed a continuous specific labelling along the surface of the fibres. The same pattern of immunoreactivity was observed in the EDL muscle stained with the anti (3-dystroglycan antibody (Fig. 3b).

In the muscles stimulated in an extended position fibres show oedema, variability of dimension, and damage. These changes were already noticed after 4h and 6h of experiment (Fig. 1b). After 24 h of stimulation several fibres of the soleus muscle were damaged; in the EDL muscle some clear signs of flogosis were evident. Infiltration with connective-tissue cells was common (Figs. 1c and 2b). In general, the changes were more pronounced in the 24 h stimulated muscles than in the 4-6 h stimulated ones and they were more pronounced in the EDL than in the soleus following the corresponding time of stimulation. The above abnormalities were seen both in the samples stained with hematoxylin-eosin (Figs. 1b, c) and in those immunolabelled (Fig. 2b). They had also been previously observed in the ultrastructure of the muscles subjected to the same experimental procedure [12].

Within the better preserved regions of the experimental muscles, numerous fibres of normal-looking shape and size were not stained, or stained only discontinuously, with the antidystrophin antibody (Fig. 2b). The above phenomenon was common in the soleus muscle stimulated in extension for 24 h and was occasionally present also in that muscle stimulated for 6 h. In the EDL muscle the said phenomenon appeared frequently, as soon as 4 h after stimulation in extension (Fig. 3c).

Several preserved fibres of experimental muscles showed lack of reaction to anti-p-dystroglycan antibody. Examination of the serial sections of these muscles, stained with antidystrophin and anti-3-dystroglycan antibody, showed that the fibres devoid of dystrophin were usually devoid of (3-dystroglycan as well (Fig. 3c, d). However,

disappearance of these two proteins seems to be not necessarily simultaneous, as is seen in the fibre marked by asterisk in Fig. 3c and 3d.

The lack of staining, or discontinuous staining, with anti-dystrophin and anti-p-dystroglycan antibody was not noticed in the control soleus and EDL muscles (Fig. 2a and 3a, b). It was occasionally seen in the muscles stimulated in a neutral position (data not shown).

Localisation of nuclei presenting DNA fragmentation

To determine whether the apoptosis plays any role in progressive damage of the muscle over-worked in extension, we studied myofibres for the presence of apoptotic DNA fragmentation by the TUNEL-method. In cryostat-sections of the normal muscles subjected to *in situ* analysis of DNA fragmentation, only few TUNEL positive nuclei were present (less than 0.01%), while in sections of the muscle stimulated for 6 h in extension some detectable increase in their number was found. The majority of the TUNEL positive nuclei laid in the interstitial tissue; a small percentage of them seemed to be localised inside the myofibres (data not shown).

After immunoreaction with an anti-laminin antibody, the connective tissue was labelled, to find out if the nuclei belong to interstitial tissue cells (surrounded by the immunoreaction), or if they are inside a myofibre. When cryostat-sections processed with the anti-laminin antibody were subjected to the *in situ* analysis of DNA fragmentation, some of the TUNEL-positive nuclei were detected inside the myofibres, in subsarcolemmal position (Fig. 4a, b). Most of the apoptotic nuclei were localised in the interstitial space (data not shown).

Discussion

In the muscle that was eccentrically electro-stimulated, according to procedure described in the Material and Methods, several fibres were damaged and necrotic (Fig. 1c and 2b). It was previously documented as well, by means of the same type of experiment [12]. In the said muscle some interesting phenomena were observed. Namely, it seems that elimination of muscle fibres takes place in the apoptotic way as well, as suggested by the

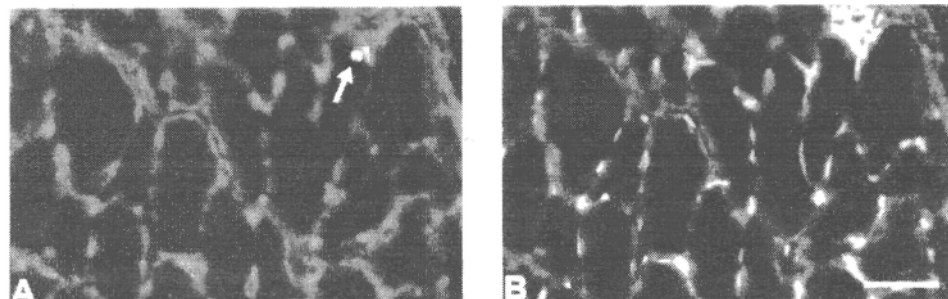


Figure 4. Soleus muscle stimulated in extension for 6 h. a) Black and white reproduction of a double labelled muscle section: the faint round areas (red in the original colour plate) are laminin-labelled basal laminae. The white indicated by an arrow is an apoptotic nucleus (yellow-green in the original colour plate), which is localised inside a myofibre. b) Double exposure to the laminin/Hoechst staining: several normal nuclei (blue in the original colour plate, white spots, are present in the field which contains the TUNEL-positive nucleus. Bar = 45 μ m.

"TUNEL-positive" nuclei, present within the muscle fibres (Fig. 4). Simultaneously, the "costamer" proteins (dystrophin and p-dystroglycan) disappeared from numerous preserved fibres of such muscle. We wonder whether the above mentioned phenomena were interconnected at all; it remains an open question.

Both the damaged muscle fibres and the lack of "costamer" proteins were observed in the EDL earlier than in the soleus. It could be explained by differences between these muscles in resistance to fatigue or in the ability of the contractile structure to contract continuously. At any rate, the cause and mechanism of the loss of dystrophin and P-dystroglycan in over-worked muscle are quite unknown; certain suggestions could be considered. One of them is, that the loss of those proteins could be due to some mechanical damage of the "costamers", accompanying the intensive contractions; the latter was already suggested by Faulkner and Brooks [6]. The damage of "costamers" could lead, in consequence, to injury of sarcolemma and to the fibre necrosis. It is possible as well that the loss of "costamer" proteins was secondary to some other cellular processes induced by over-working of the over-extended muscle. Another possibility, already suggested in the Introduction, is that the loss of the "costamer" proteins could accompany some reorganization of the contractile structure and reorganization of its connections to the extracellular matrix. This process could destabilise the sarcolemma and cause its increased sensitivity to injury. Therefore, any serious injury to sarcolemma, with all the negative consequences to the muscle fibre, could only take place in extreme situations, when any chance of adaptation is out of question. Such "physiological" interpretation of the loss of "costamer" proteins seems to be in agreement with the well-known fact, that the lack of dystrophin does not necessarily bring about any fibre injury. In the Duchenne dystrophy, for example, not all groups of muscles are affected and not all fibres within the affected muscles get seriously injured; in the female carriers of Duchenne dystrophy and in the *mdx* dystrophy the muscle fibres usually remain preserved. Also Miyazato and co-workers observed the prolonged absence of dystrophin in the surviving myocytes in the injured heart muscle [15]. In contradiction, Vater et al. [21] found that the loss of dystrophin precedes the necrosis of fibres in the muscle injured by the toxin. However, no matter what the reasons were, the loss of "costamer" proteins and the possible destabilisation of sarcolemma, could explain the well-known fact of the late injury to the fibres in the muscle overworked in extension and the late increase in level of the muscle enzymes in the blood (2nd-3rd day after over-working).

Whether at all, and in what way, can the loss of dystrophin and p-dystroglycan predispose the muscle fibres to necrosis, or to death by apoptosis, need some more detailed examination. All the above mentioned questions are under our study now.

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