Caveolae in the Muscle Overworked in an Extended Position
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Abstract
The amount of caveolae increased in the rat leg muscles while electro-stimulated in extension for some hours. In muscles stimulated for the same period in neutral position, or held in extension without stimulation, the amount of caveolae was comparable to the control level; it increased only while the experiments lasted longer. Thus, eccentric contraction accelerates the formation of caveolae in muscle fibres.

Key words: caveolae, dystrophin, dystrophy, eccentric contraction, electro-stimulation, sarcolemma, skeletal muscle.

Skeletal muscle which contracts in an extended position gets damaged much easier than a muscle overworked in a neutral or in a shortened position. In such a muscle pain, stiffness, motor impairment and dysfunction appear with concomitant elevation of the muscle enzymes in serum, as a sign of impairment of the muscle fibre membrane integrity. Damage and necrosis of muscle fibres are quite common. The reason for the muscle fibre injury following eccentric contraction is unknown so far. Several hypotheses have been put forward, concerning both metabolic and mechanical reasons, including disruption of the cytoskeletal network and injury of sarcolemma or of other muscle fibre membranes. However, no convincing evidence has been found so far revealing the primary cause of muscle damage occurring after eccentric contraction. On the other hand, the moderate eccentric contraction is more effective for muscle adaptation than muscle contraction in neutral or shortened position [14, 15].

As recently found [3, 4], in the muscle overworked in an extended position, numerous fibres of normal-looking shape and size lose dystrophin from the sarcolemma region, similar to that in the Duchenne or mdx muscular dystrophy [1]. Those muscles also lose (to a lesser degree) other proteins of the sarcolemmal region: proteins of the dystrophin-associated complex (DAP) and spectrin [4]. The loss of those proteins was observed also in the Duchenne muscular dystrophy [7]. Besides the loss of dystrophin and the DAP proteins from the sarcolemma region, [4] several other clinical and metabolic features are quite similar in the dystrophin-deficient dystrophic muscle and also in the muscle following eccentric contraction [14, 15].

Dystrophin and associated proteins form at sarcolemma a network of continuous transversal structures, with longitudinal interconnections, called costameres. The costameres surround the Z- and M- lines for the entire length of the muscle fibre [17]. Several other cytoskeletal proteins appear to be associated with the costameres, for instance, spectrin, α-actin, actin and talin, but also some non-cytoskeletal proteins [2]. The costameres of the striated muscle appear to link the contractile apparatus to the extracellular matrix, as well as being a scaffold for the topological localization of signaling proteins [2, 17]. Caveolin-3, the muscle-specific protein of caveolae, is colocalized in sarcolemma with the DAP complex [16], but not as its integral component [5].

As documented within the past few years, caveolae, the 50-100 nm vesicular invaginations of the plasma membrane, represent its specialized microdomains. They are found in most types of cells including the muscle fibres [12]. Caveolae are dynamic structures which participate in a number of functions, including vesicular trafficking events and signal transduction. The main structural and integral proteins of caveolae are the caveolins whose oligomers form a structural framework playing an important role in the formation of caveolae membranes. The major lipid components of caveolae membranes are cholesterol and sphingolipids [11, 12]. The caveolins interact with several proteins (with those containing the caveolin-binding sequence motives) as G-proteins, some kinases, growth factor receptors or NOS.
Caveolins usually function as their inhibitors [12]. In this paper we present the increased number of caveolae within the sarcolemma region of the muscles which were overworked in extended position.

**Materials and Methods**

Three-months-old female albino Wistar rats were used. The slow soleus (SOL) and the fast extensor digitorum longus (EDL) muscles were maintained in extended position by immobilization of the ankle joint, or by cutting the synergistic muscles. Stimulating electrodes were implanted on the sciatic nerve and that nerve was continuously stimulated from 2 hours to 4 days by pulses of 0.3 ms duration and 20 Hz frequency. All procedures were applied as previously described [3, 8]. As controls muscles were used from untreated animals of the same population, the muscles immobilized in extension without stimulation, and the muscles stimulated without joint immobilization. In each group 2-4 animals were used, with some series of experiments repeated several times. From each muscle several (minimum 3) samples were examined.

Immediately after decapitation (under ether anesthesia) muscle was excised, as described in detail [10] and immediately subjected to further procedures.

For the study of ultrastructure all procedures of muscle fixation, embedding and sample preparation were applied as previously described in detail [8, 10]. Ultrathin sections were prepared on an LKB Ultratome R III; they were inspected using JEM-100 B and JEM 1200 EX electron microscopes.

For immunohistochemical study all procedures were performed as previously described [4]. Monoclonal antibodies against dystrophin (1:300) and β-dystroglycan (1:50) were used (purchased from Novocastra, Newcastle upon Tyne, UK). For immunohistochemical analysis an epifluorescence microscope (Carl Zais, Oberkochen, Germany) was used.

**Results and Discussion**

As found in the present work (and previously as well [3, 4]), in a muscle stimulated in an extended position there were certain regions severely damaged, containing necrotic fibres, while some other regions were nearly normal. In the ultrastructure, of those muscles disruptions of sarcolemma were found in some fibres with concomitant fibre swelling, post-mortem-like changes of the contractile apparatus and damage of mitochondria, as previously observed as well [9]. However, numerous muscle fibres were preserved, with normal-looking mitochondria and containing a lot of polysomes.

Within some better preserved parts of the experimental muscles, numerous fibres of normal-looking shape and size were not stained on their surface, or stained only discontinuously, with anti-dystrophin antibody (Fig. 1b). The same fibres often lose staining (to a lesser degree) with β-dystroglycan antibody (Fig. 1c), and also with α-sarcoglycan, γ-sarcoglycan and spectrin antibodies [4]. Such fibres were found after 4-24 h, but not after 2 h, of experiment, in the soleus and EDL muscles.

![Figure 1. SOL muscle immunolabelled with anti-dystrophin and anti-β-dystroglycan antibody: transverse sections.](image)
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They have never been found in the control intact muscles, nor in the muscles maintained in extension without stimulation. However, they have occasionally been seen in the muscles stimulated in a neutral position, but only in the damaged regions [4 and data not shown].

The electron-microscopic examination of the control muscles showed the well-known regular structure of muscle fibres. Vesicles of round or elliptic shape and about 50-100 nm in size were occasionally found in the sarcolemma region. The connection was seen between the interior of those vesicles and the extracellular space (Fig 2a). Such structures correspond to those mentioned in introduction and known as caveolae [11, 12]. They seem to have been more frequent in the control soleus muscle than the control EDL, and more frequent within regions of sarcolemma close to the Z-line and I-band, than at the A-band level (Fig. 2b,c). The caveolae became very numerous following muscle stimulation in an extended position. This phenomenon was observed within well preserved fibres, containing a lot of normal-looking mitochondria and polysomes, already after 2 h of stimulation in extension, being very intensive after 5-8 h.

Figure 2. Electron micrographs of the studied muscles. a) Control SOL muscle, longitudinal section. Caveolae in satellite cell are shown by arrows. b) Control SOL muscle, longitudinal section. A-band (A), I-band (I), Z-line (Z). Caveolae are shown by arrows. c) Control EDL muscle, longitudinal section; caveola is shown by arrow. d) SOL muscle stimulated in extension for 5 h, transverse section. Numerous caveolae are seen along the sarcolemma; those which connection with the extracellular space is visible are indicated by arrows. e) SOL muscle stimulated in extension for 6h, transverse section of satellite cell (S) region. Numerous caveolae are present within muscle fibre (regions indicated by arrows). f) SOL muscle stimulated in extension for 5h, longitudinal (some oblique) section. Very numerous caveolae are present along the folded sarcolemma. Bar = 0.15 \( \mu \)m (a); 0.30 \( \mu \)m (b,c,d); 0.40 \( \mu \)m (e,f).
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(Fig 2 d-f), and still present after 3 days of stimulation.

In the muscles stimulated for 6 hours in a neutral position, as well as in the muscle maintained in extension without stimulation for 6 h (Fig 3a,b), the frequency of caveolae were comparable to that in the control untreated muscles (Fig 2b,c). It suggests that the increased formation of caveolae during some hours is only induced when the muscle is simultaneously overstretched and overworked. Contrary to the short-time effect, when the experiments lasted longer (some days), the caveolae became frequent also in the muscles stimulated in a neutral position and in those extended without stimulation (Fig 3c,d). It allows us to conclude that though the increased formation of caveolae is not a specific reaction of the muscle overworked in extension, yet this reaction gets accelerated in such a muscle. The overworking and the extension, applied to the muscle as a separate stimulus, also induced the caveolae formation, but only some time later, and perhaps to a smaller extent than while acting together. The increased number of caveolae was also found in the regenerating muscle [20]. Thus, the increased formation of caveolae is perhaps connected with the muscle fibre adaptation/recovery/regeneration processes. If it is so, the accelerated induction of caveolae formation in the eccentrically contracting muscle could be connected with the well-known better adaptive effect of such contraction than any other kinds of muscle activity.

The increased number of caveolae and the elevation of caveolin-3 were found in the Duchenne and mdx dystrophic muscles [13, 18]. Thus, our observation proved that the next feature of similarity exists between the normal muscle when overworked in extension and the dystrophic muscle. However, in the dystrophic muscle the increased number of caveolae was supposed to be a pathogenic event of dystrophy [13]. The presented results rather exclude such possibility. Additionally, the genetic defect of caveolin-3, and its absence from the sarcolemma region, was recently identified as that causing the autosomal dominant limb girdle muscular dystrophy (LGMD-1C) [6]. It means that the lack, but not the excess, of caveolin-3 in the sarcolemma region is the

Figure 3. Electron micrographs of the studied muscles. a) SOL muscle maintained in an extended position without stimulation for 6h, longitudinal section. In two adjacent fibres amount of caveolae seems to be comparable with that in the control-intact SOL muscle (some caveolae are indicated by arrows). b) SOL muscle stimulated for 6h in a neutral position, transverse section; the satellite cell (S) region. Few caveolae present in the muscle fibre are shown by arrows. c) EDL muscle maintained in an extended position without stimulation for 4 days, longitudinal section. Numerous caveolae and vesicular structures are present in two adjacent fibres; some caveolae are indicated by arrows. d) SOL muscle stimulated in neutral position for 3 days; satellite cell (S) region in a transverse section of the muscle fibre. Numerous caveolae and caveolae-like structures are present in muscle fibre (regions shown by arrows). Bar = 0.30 μm (a,d); 0.50 μm (b); 0.17 μm (c).
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pathogenic event of dystrophy. On the other hand, the loss of caveolae was observed early after exposure of muscle to bupivacaine [19, 20] which is well-known to induce necrosis of skeletal muscle fibres. All data taken together may suggest that the increased amount of caveolae is connected with the muscle fibre viability, and that in the dystrophin-deficient muscle the increased amount of caveolae could appear as a recovery or compensatory phenomenon.

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