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Morphological adaptation and protein modulation of myotendinous junction following moderate aerobic training

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Summary. Myotendinous junction is the muscle-tendon interfacethrough which the contractile force can be transferred from myofibrils to the tendon extracellular matrix. At the ultrastructural level, aerobic training can modify the distal myotendinous junction of rat gastrocnemius, increasing the contact area between tissues. The aim of this work is to investigate the correlation between morphological changes and protein modulation of the myotendinous junction following moderate training. For this reason, talin, vinculin and type IV collagen amount and spatial distribution were investigated by immunohistochemistry and confocal microscopy. The images were then digitally analyzed by evaluating fluorescence intensity. Morphometric analysis revealed a significant increased thickening of muscle basal lamina in the trained group (53.1±0.4 nm) with respect to the control group (43.9±0.3 nm), and morphological observation showed the presence of an electron-dense area in the exercised muscles, close to the myotendinous junction. Protein concentrations appeared significantly increased in the trained group (talin +22.2%; vinculin +22.8% and type IV collagen +11.8%) with respect to the control group. Therefore, our findings suggest that moderate aerobic training induces/causes morphological changes at the myotendinous junction, correlated to the synthesis of structural proteins of the muscular basal lamina and of the cytoskeleton.

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Introduction

The myotendinous junction (MTJ) is a specialized interface between muscle and tendon which transmits the contractile force, generated by myofibrils and the elastic force stored in the connective tissue during movement (e.g. epimysium, perymisium, endomysium) (Polican Ciena et al., 2012; Turrina et al., 2013). At morphological level, MTJ shows tendon interdigitations which penetrate into the muscle mass, parallel to myofibril direction in fusiform muscles. Their presence increases the contact surface between tissues and allows the MTJ to resist muscle contractile forces that are in the range of 1.8 to 3.5×10^4 N/m^2 (Tidball and Chan, 1989; Kojima et al., 2008). The MTJ structure is rather complex and is still the object of numerous studies. It depends on the functional and structural features of both tissues. The 3D reconstruction of human MTJ reveals the presence of a tendon meshwork, which forms structures resembling ridge-like protrusions. The myofibrils continue in the ridge-like protrusions and connect to the tendon tissue (Knudsen et al., 2014). In addition, rat MTJ between the anterior belly of digastric muscle and the intermediate tendon shows classical morphologic characteristics, but presents atypical regions correlated to a sort of microtendons (Ciena et al., 2012). At MTJ level, the collagen fibril-rich matrix binds the muscular basal lamina proteins, which, in turn,

link the actin microfilaments that extend from the Z-line in the proximity of the sarcolemma, through specific protein complexes (Charvet et al., 2012).

The interaction between muscle fibers and tendon extracellular matrix (ECM) is characterized by two types of protein linkage structures: the dystrophin-glycoprotein complex (DGC) and the vinculin-talin-integrin system (Anastasi et al., 2009). The DGC is composed by three sub-complexes: the sarcoplasmic (SSC), the dystroglycan (DSC) and the sarcoglycan transmembrane (STSC) sub-complex. The key intracellular protein of SSC is dystrophin, which links F-actin, dystrobrevin (α, β), syntrophin (α, β1, β2, γ1 and γ2), syncoilin, dysbindin, synemin and ß-dystroglycan. The dystrophin/ß-dystroglycan bond forms the point of junction between the intracellular domain and the sarcolemma. The DSC consists of α-dystroglycan (extracellular protein) and ß-dystroglycan (trans-membrane glycoprotein). α-Dystroglycan is able to bind ß-dystroglycan, in the sarcolemma, and laminin and agrin, in the extracellular domain. Finally, the STSC composition is represented by the association of sarcoglycans (α, β, γ, δ, ε) and sarcospan. This sub-complex associates with DSC and stabilizes it (Groh et al., 2009). In the vinculin-talin-integrin system, integrins are αβ heterodimeric receptors that mediate cell attachment to the ECM. Actin filaments of terminal sarcomeres bind to talin, α-actinin and vinculin, while integrin β subunit peptides bind only to talin and α-actinin. At the extracellular domain, α7β1 integrins, which are particularly abundant at the MTJ level, link laminin 211 (Anastasi et al., 2004). Then, in both protein linkage complexes, α7β1 integrins and dystroglicans link laminin 211, which is one of the muscle basal lamina proteins. The basement membrane is also composed of type IV collagen, entactin, perlecan, agrin and decorin (Hohenester and Yurchenco, 2013).

The MTJ is a dynamic interface which can be modified by numerous physiological and pathological conditions (Roffino et al., 2006; Baudry et al., 2012). In a previous work, we demonstrated that moderate aerobic training leads to changes in the MTJ ultrastructure. In particular, it has been observed that the surface where muscle and tendon interconnect is increased, thanks to a higher number of bifurcations and of branched interdigitations of the tendon (Curzi et al., 2012). Furthermore, we showed that the MTJ may also be modified by resistance exercise, demonstrating that resistance training prevents the interface loss generated by disuse atrophy and increases the tendon process extensions penetrating into the muscle (Curzi et al., 2013).

The literature reports much evidence of modulation of the MTJ proteins in particular conditions, mostly regarding the vinculin-talin-integrin system, and some of these highlight the possible role of physical exercise. In fact, talin and vinculin concentrations increase following eccentric contractions in mice soleus and plantaris muscles (Frenette and Côté, 2000) and following maximal resistance exercise, after bed rest, in human vastus lateralis (Chopard et al., 2005). Moreover, α7β1 integrin expression appears significantly increased after running training in rat soleus muscle (Kääriäinen et al., 2001).

Mechanical loading also increases the expression of ECM proteins at this level, such as tenascin-c, an elastic glycoprotein, binding other ECM proteins as well as cell membrane adhesion receptors. (Järvinen et al., 2003). Furthermore, the concentration of type IV collagen, the main component of muscular basal lamina, can be increased by training protocols. In the white fibers of the quadriceps femoris muscle, type IV collagen concentration increases after acute exercise (Koskinen et al., 2001). In patients with knee osteoarthritis, an increment of type IV collagen has been observed in vastus lateralis muscle, after 12 weeks of resistance training (Mattiello-Sverzut et al., 2013).

In the following research, we investigated MTJ adaptations to moderate aerobic training, analyzing the correlation between protein composition and morphological changes. In particular, we evaluated the ultrastructural modifications of the MTJ in trained and control rats, and we investigated the protein distribution and concentration of talin, vinculin and collagen IV in both groups by immunolocalization and confocal microscopy.

Materials and methods

Animals and experimental procedures

Eight male Sprague-Dawley rats, aged 8 weeks, were placed in individual cages and fed a standard diet without limitations. Room temperature (R.T.) was kept at 20.5±0.5°C and 12 h of light were automatically alternated with 12 h of dark. After 1 week of acclimatization, 4 rats were randomly chosen to run on a six-lane rodent treadmill three times a week. Time and speed were gradually increased to reach 1 h a day (3 times a week), 25 m/min in 5 weeks; these parameters were maintained constant for a further 5 weeks of training (Frizziero et al., 2011). The level of aerobic training thus obtained approximately corresponded to 65-70% VO_{2max}, according to Wisloff et al. (2001). At the end of the 10-week training, control and trained animals were euthanized under general anaesthesia (ketamine 87 mg/kg and xylazine 3 mg/kg - with i.v. injection of Tanax). Animal handling, training protocol and mode of killing were approved by the Ethical Committee of Rizzoli Orthopedic Institute, according to the European and Italian Laws on animal experimentation and to the principles stated in the "NIH Guide for the Care and Use of Laboratory Animals".

Transmission electron microscopy (TEM)

Gastrocnemius muscles were obtained from both hind legs, quickly dried and freed of connective tissue.

The muscles from the right hind-limbs were processed for electron microscopy. Samples, maintained under tension with pins, were immediately fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer for 6 h and quickly minced into smaller (<1 mm^3) fragments. They were then post-fixed with 1% OsO_4 for 1 h in the same buffer, dehydrated with alcohol, and embedded in araldite. Semithin sections were obtained in order to be able to study a thorough longitudinal plane of the muscle fibres, allowing a sharp MTJ identification, and successively stained with 1% toluidine blue in distilled water at 60°C. Thin sections, stained with uranyl acetate and lead citrate, were observed with Philips CM10 electron microscope (Burattini et al., 2013).

Immunofluorescence

The gastrocnemius from the left hind-limbs were fixed by overnight immersion in 4% paraformaldehyde and 0.1 M phosphate buffer saline (PBS), pH 7.4. After a brief wash in PBS, tissues were immersed in sucrose (30% w/v; with 0.1% sodium azide) and were then embedded in Tissue-Tek (Sakura Finetek Europe, Zoeterwoude, The Netherlands) and immediately frozen in liquid N_2-cooled isopentane. For immunofluorescence analysis, adjacent serial sections (12 μm) were cut and collected onto polylysine coated glass slides. Immunofluorescence techniques were carried out directly on microscope slides. Samples were permeabilized with 0.25% Triton X-100 in PBS, for 10 minutes at R.T., and rinsed again with PBS. Samples were treated with 2% bovine serum albumin (BSA), 5% normal goat serum (NGS) in PBS (PBS-BSA-NGS mixture), for 30 minutes at R.T., and then incubated with mouse primary antibodies against talin (Sigma-Aldrich; 1:200 in the PBS-BSA-NGS mixture), rabbit primary antibodies against vinculin (Sigma-Aldrich; 1:200 in the PBS-BSA-NGS mixture) and rabbit primary antibodies against collagen IV (Millipore; 1:100 in the PBS-BSA-NGS mixture), overnight at 4°C.

The next day, samples were rinsed with PBS and incubated with a FITC-conjugated goat anti-rabbit secondary antibody (Millipore; 1:500 in the PBS-BSA-NGS mixture), or with a CY3-conjugated goat anti-mouse secondary antibody (Millipore; 1:500 in the PBS-BSA-NGS mixture), for 1 h at R.T. in the dark. Control sections for non-specific staining were subjected to the same incubation protocol but with the primary antibody omitted.

Slices were finally mounted with the Vectashield mounting media for fluorescence (Vectorlabs).

Confocal laser scanning microscopy (CLSM)

Images were analyzed by means of a Leica TCS-SP5 confocal microscope, connected to a DMI 6000 CS inverted microscope (Leica Microsystems CMS GmbH) and analyzed using the software Leica Application Suite Advanced Fluorescence (LAS AF). Samples were examined using an oil immersion objective lens (40x N.A. 1.25). Excitation was at 488 nm (FITC) and 543 nm (CY3); emission signals were detected at 519 nm and 598 nm, respectively. CLSM images are presented as single-plane images and image analysis has been carried out using the *ImageJ* software (Salucci et al., 2013).

Morphometric and statistical analysis

Measurements of muscle basal lamina thickening were carried out only along the tendon interdigitations which are parallel to the longitudinal axis of the myofibrils. One hundred measurements were evaluated for each muscle, therefore four hundred for each group.

The relative amounts of proteins present at myotendinous junctions were estimated using digital analysis of confocal images. White light micrographs were used to identify the MTJ in each sample; serial images were examined, and the image halfway between the top and bottom of the labeled MTJ was chosen for quantitative analysis. This area was chosen to eliminate possible variations in signal intensity due to the inclusion of images of the top or bottom surfaces of a muscle fiber (Douglas et al., 1994). Fifty images for each group and for each protein were analyzed. The intensity of the fluorescent signal was measured, counting the percentage of stained area at the MTJ level (Edwards and Launikonis, 2008; Ambrogini et al., 2014). The basal lamina thickening and the fluorescence intensity were measured in semiautomatic mode, using ImageJ analysis software. All data were expressed as mean values ± SEM. Differences in basal lamina thickening and fluorescence intensity of proteins between the two groups were determined using Student's t-test. Statistical significance was set at $P<0.05$.

Results

Morphological and morphometric analysis of MTJ ultrastructural changes

The MTJ micrographs, obtained from both groups, revealed interesting morphological differences. In agreement with our previous observations (Curzi et al., 2012), the muscle-tendon interface appeared increased in the trained group, due to ultrastructural adaptations. In fact, MTJs of trained rats displayed tendon interdigitations which penetrated deeper into the muscle mass than in the sedentary rat MTJs, and some bifurcated finger-like processes were observed following exercise training (Fig. 1A,B).

At high magnification, in the trained group, the muscle basal lamina along the tendon interdigitations showed a remarkable thickening (Fig. 1C,D). The morphometric analysis revealed that exercise protocol produced a significant 17.33% increase in the thickness mean value of basal lamina (control = 43.9±0.3 nm; trained = 53.1±0.4 nm; Fig. 1E). Furthermore, in close

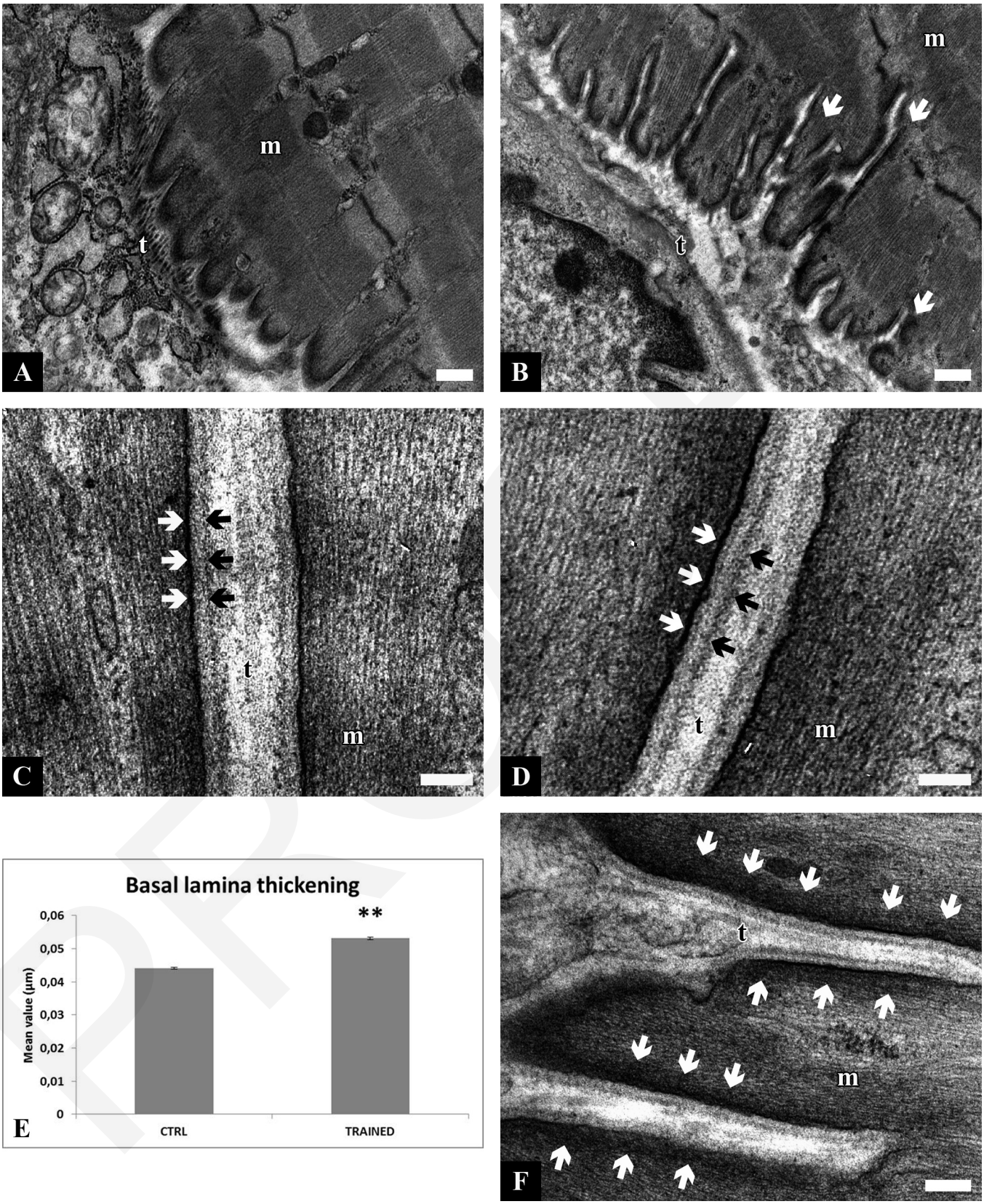

Fig. 1. TEM micrographs of gastrocnemius MTJ from CTRL **(A,C)** and TRAINED **(B, D, F)** groups. The muscle-tendon (m, t) interface of exercised group **(B)** reveals an increased extent of MTJ folding respect to the CTRL rat **(A)** and the presence of bifurcated tendon processes. Along the interdigitations, the muscular basal lamina of TRAINED rats **(D)** appears more thickened than in the CTRL group **(C)**. Morphometric analysis **(E)** confirms this significant thickening (CTRL 43.9±0.3 nm; TRAINED 53.1±0.4 nm); ** t-test $p<0.01$. In the exercised rat muscle, an electron-dense area close to MTJ is observed along the tendon processes **(F)**. Bars: A, B, 0.5 μm; C, D, 0.1 μm; F, 0.2 μm.

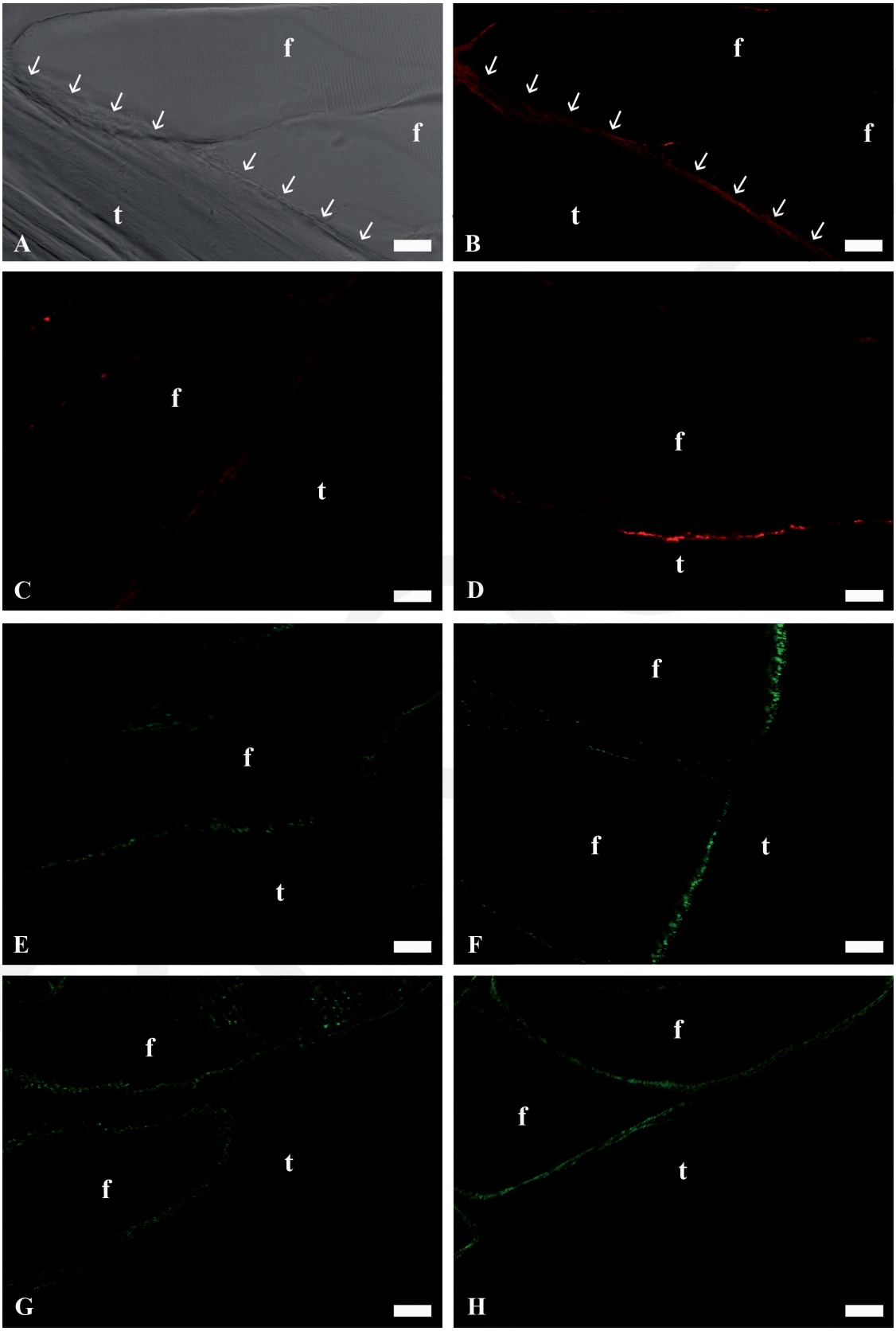

Fig. 2. Light microscopy CLSM **(A)** and talin dark field CLSM **(B)** of the same MTJ. Immunofluorescence analysis of talin **(C, D)**, vinculin **(E, F)** and collagen IV **(G, H)** in MTJ longitudinal sections of CTRL **(C, E, G)** and TRAINED **(D, F, G)** rats (f: muscle fiber; t: tendon). Bars: 10 μm.

proximity to MTJs, enlarged electron-dense areas were observable in the trained muscle tissue, especially along the tendon interdigitations (Fig. 1F). These dark areas suggested the possible presence of dense protein complex.

Immunofluorescence and morphometric analysis of protein variations at the MTJ level

To further investigate ultrastructural modifications, gastrocnemius MTJs of control and trained rats were studied by means of immunofluorescence analysis. Confocal microscope images (Fig. 2) and morphometric analysis (Fig. 3) clearly demonstrated the presence of protein adaptations following moderate training. Figures 2A and 2B showed the same MTJ at light microscopy CLSM (A) and at dark field CLSM (B), to precisely identify MTJ talin immunolocalization. Talin and vinculin staining (Fig. 2C-F) revealed a different protein distribution between groups. In fact, in the control group, the staining appeared discontinuous and punctiform, while in the trained group it seemed more homogeneous.

These observations were confirmed by fluorescence intensity analysis (Fig. 3A-B). In fact, both cytoskeletal proteins increased in the trained group in comparison to the control, respectively by 22,2% and 22,8%. The type IV collagen staining (Fig. 2G-H) also increased in the exercised group by 11,8% (Fig. 3C), suggesting that moderate aerobic training did not only modulate the muscle fiber cytoskeleton but also the muscle basal lamina.

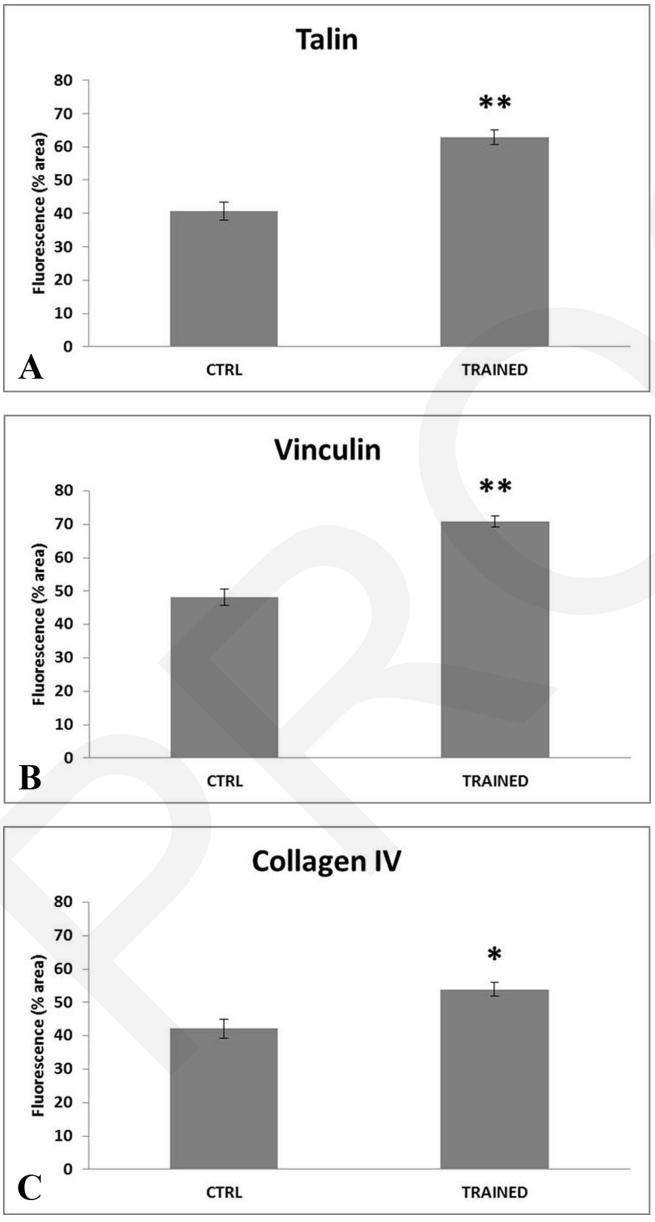

Fig. 3. Analysis of talin **(A)**, vinculin **(B)** and collagen IV **(C)** fluorescence intensity at the MTJ level. * t-test p<0.05.

Discussion

The MTJ is a key interface for the locomotor system, where the muscle force is transmitted to its tendon and therefore to bone tissue through enthesis. For its physiological role, the MTJ is susceptible to morphological modifications, in particular when physiological or pathological conditions change tendon or muscle structure, or the intensity of the strength expressed by the latter (Charvet et al., 2012). Present data confirm previous findings concerning morphological adaptations generated by moderate training (e.g. the presence of longer finger-like processes and the numerical increase of bifurcated interdigitations in MTJ from trained animals) (Curzi et al., 2012); in addition, they report the presence of a morphological modulation of muscle basal lamina, which appeared thickened in the exercised rat muscles at the MTJ level, highlighting ultrastructural modifications in both the tendon and the muscle tissue.

Furthermore, by investigating the protein composition of the MTJ, our data clearly show the occurrence of protein adaptations in trained muscles, suggestive of an intense protein synthesis. In particular, the increased talin and vinculin concentrations imply a modulation of cytoskeleton protein complexes necessary for the link between muscle and tendon. In the literature, the increased expression of these proteins following an eccentric exercise protocol had been hypothesized to be associated with serial sarcomere addition and muscle repair (Frenette and Côté, 2000). Our results are partially consistent with this hypothesis: in fact there is no evidence which correlates moderate endurance training with sarcomerogenesis, but the protein synthesis may be correlated with muscle structure adaptations generated by exercise. The protein modulation may help in strengthening the MTJ structures, in order to support the

new condition and to prevent possible damage (Frenette and Tidball, 1998). This idea appears to be indirectly confirmed in mdx mice, where, concomitant with the lack of dystrophin, the protein concentration of the vinculin-talin-integrin system is significantly increased (Law et al., 1994). As previously described, DGC and vinculin-talin-integrin system are different complexes through which muscle makes contact with tendon. The above described adaptations in the expression of cytoskeletal proteins occurring in mdx muscles could be compensatory for dystrophin absence, as they contribute to strengthening the tissue interface (Spassov et al., 2012).

In the gastrocnemius MTJ of trained rats an increased concentration of type IV collagen, the principal component of muscular basal lamina, was also found. Changes in type IV collagen expression in fast-twitch muscle are in agreement with adaptations in myofibrillar protein synthesis during loading. Both protein modifications are related to changes in muscle strength and motor activity (Kaasik et al., 2011). A sensible increase of type IV collagen expression may be related to the increased capillary density following running training (Kvist et al., 1995). However, in our study no significant vessel numerical increase was appreciated in exercised condition, both at immunofluorescence and ultrastructural approaches.

In conclusion, morphological adaptation and protein modulation of gastrocnemius MTJ are observed following moderate endurance training. The morphological changes affect both the tendon and the muscle tissue, and the latter also displays cytoskeletal protein modifications. These changes could be explained as adaptations to the increased tension induced by exercise, aimed at strengthening the MTJ structure. This idea appears to be indirectly confirmed by the opposite results on the MTJ following immobilization, described by Kannus et al. (1992). In fact, they revealed a decreased contact between muscle and tendon after 3 weeks of immobilization.

These findings allow a better understanding of role and function of MTJ proteins, which presently are also the object of intense studies in a number of myopathies. Moreover, our data concerning the physiological effects of moderate endurance training on the MTJ appear to support its role in rehabilitation following muscle injury. In fact, we demonstrate here the capacity of this exercise protocol to modulate not only muscle and/or tendon but also the key interface between tissues, necessary for the contractile force transmission.

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