Morphological changes of myotendinous junction generated by muscle disuse atrophy

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Summary
The skeletal muscle contraction is transmitted to the tendon through the myotendinous junction (MTJ), where the proximal extremity of the tendon forms characteristic finger-like processes, penetrating into the muscle mass. We recently demonstrated that changes at MTJ level occur as an adaptation to exercise-induced tension increase, allowing a better tension resistance. The aim of this study is to analyse MTJ behavior in disuse condition, furtherly investigating the strict morpho-functional correlation. Therefore, 4 hind-limb suspended (HS) and 4 control (CTRL) rats were studied. After sacrifice, MTJs of plantaris muscle were processed for electron microscopy. After 5 days of suspension, skeletal muscle displays signs of atrophy in response to decreased mechanical loading. In fact, close to muscle-tendon interface, irregular misaligned sarcomeres, with absent Z-lines, were observed. Muscle intermyofibrillar component spreads, especially between terminal filaments and tendon finger-like processes. The surface area between muscle and tendon was evaluated with P/B ratio, where "B" is the base and "P" is the interface length of tendon finger-like processes at MTJ level. After 5 days of suspension, the IL/B ratio decreases from 6.39 in CTRL to 3.92 in HS and the suspension reduces the mean extension of finger-like processes from 1.84 µm to 0.97 µm.
In conclusion, along with muscle atrophy features, ultrastructural changes occur at the MTJ organization level, as an adaptation to muscle unloading.

Key words: Myotendinous junction, ultrastructure, suspension, atrophy, disuse.

Introduction

The myotendinous junction (MTJ) is a specialized anatomical region of the skeletal system, where the tension generated by muscle contraction is transmitted to extracellular connective tissue of the tendon (ECM) (Ciena *et al.*, 2010), so representing the largest area of force transfer in skeletal muscle (Tidball, 1991). At the ultrastructural level, the proximal extremity of the tendon forms finger-like processes that penetrate into the muscle mass. An interdigitated profile indicates the separation between tissues, where a sort of muscle-tendon crosstalk occurs. Membrane folding increases the contact area between muscle fibers and tendon collagen fibers (from 10 to 50 times) (Tidball and Lin, 1989). The morphology of the interface between a muscle fiber and the tendinous connective tissue looks like an adhesive joint (Trotter, 1993).

Structurally, actin filaments extend into the terminal muscle cell processes at the MTJ level and are bundled into a subsarcolemmal dense plaque that is also thought to be a specialized area for adhesion to and force transmission across the cell membrane (Law and Tidball, 1993). The molecular organization of dense plaque is believed to be analogous to the focal adhesion sites of cultured cells.

The extracellular domains of transmembrane integrins bind to ECM proteins and the cytoplasmic ones associate with a complex of cytoskeletal proteins connected to actin filaments.

Vinculin is a prominent cytoskeletal protein

highly concentrated at MTJ, that can interact with other cytoskeletal proteins, including α-actinin and talin. Talin is also a protein mostly localized at the cytoplasmic face of cell-matrix junction and is ubiquitously involved in the linkage of cytoskeletal elements to the cell membrane at MTJ: it contains binding sites for β1 integrin, α-actinin, and vinculin.

Vinculin has been proposed to strengthen connections between integrins and actin filaments to support force transfer from the cytoskeleton to the ECM (Opazo Saez *et al.*, 2003). At the extracellular domains, integrins are the principal plasma membrane receptors for binding ECM components, including collagens, fibronectin, vitronectin and laminins (Kato *et al.*, 2007).

The connection between ECM and integrins are important for transmitting mechanical forces and for maintaining skeletal muscle fibers. Accordingly, defects in integrin function lead to muscle fiber degeneration: mutations in the gene encoding the α7 integrin subunit cause congenital myopathy in humans (Hayashi *et al.*, 1998), and genetic ablation of either the α5 or α7 integrin subunits causes muscular dystrophy in mice (Mayer *et al.*, 1997; Taverna *et al.*, 1998).

For its function, the MTJ is susceptible to ultrastructural modifications, in particular physiological and pathological muscle conditions. In fact, in literature, several studies confirmed that, for example, spaceflight can lead to a rapid and large decrease in MTJ area (Tidball and Quan, 1992). In atrophic human muscle, undergoing amputation in the distal or proximal third of the lower leg, the contact between muscle and tendon is reduced by quantitative and qualitative changes in the myotendinous endings (De Palma *et al.*, 2011). Furthermore, extensive collagen deposition adjacent to the MTJ, alterations in the length and shape of the finger-like processes, thickened sarcoplasmic invaginations and central communications with lateral junctions appear with increasing age in rat (Polican Ciena *et al.*, 2012).

In particular, in a recent work we demonstrated that MTJ could be modified, in gastrocnemius and extensor digitorum longus (EDL) rat muscles, by a particular resistance training protocol that increased the percentage of the branched tendon interdigitations and their bifurcation mean. Then we confirmed that MTJ can adapt to muscle changes induced by exercise (Curzi *et al.*, 2012).

In this work, for experimental procedures, we used protocols previously demonstrated to generate evident muscle modification. Several studies have confirmed how the leg muscles of rats suspended for 5 days show clear signs of muscle atrophy. In fact, an ultrastructural disarrangement, such as disorganization of myofilaments, misalignment of adjacent sarcomeres and distortion or absence of Z lines have been observed (Kim *et al.*, 2007).

The aim of this work is to further highlight the muscle ultrastructure in the atrophic condition, generated by disuse, and to investigate the MTJ behavior during unloading.

Materials and Methods

Animals and experimental procedures

Eight male albino Sprague-Dawley rats were housed singly in Plexiglas suspension cages on a standard 12:12-h dark-light cycle in a room maintained at 24±1°C. Food and water were provided ad libitum. Animal care and use were in accord with the "Ames Research Center Animal Users Guide" (AHB 7180). Rats were assigned randomly and equally to one of two groups: 1) control (CTRL) and hind-limb suspended (HS). The rats were suspended for 5 days, the tail was cleaned with ethanol and sprayed with a benzoin-isopropyl alcohol mixture to protect from irritation. The benzoin-alcohol was dried using a hair dryer. Approximately two-thirds of the tail was then wrapped in a piece of Fast-Trac adhesive tape, covered with a stockinette and secured with fiber tape. The Fast-Trac tape was passed through a wire hook, which was then suspended from a fishing swivel. The swivel was, in turn, suspended from the overhead track system. This arrangement allowed the rats to move freely about the cage on their forelimbs. The suspension tracks were blocked, so that the rats were unable to touch the sides of the cage with their hind-limbs (Grindeland *et al.*, 1994; Linderman *et al.*, 1994).

Transmission electron microscopy

The rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (50 mg/kg body wt) and weighed. The right plantaris muscles were quickly freed of connective tissue and weighed on a precision scale. Samples were tied to an applicator stick at physiological length and

immediately fixed with 1.4% glutaraldehyde in a 0.2 M sodium cacodylate buffer at pH 7.2 for 1 h at 4°C. The muscles were then rinsed in cacodylate buffer and minced into small bundles (<1 mm^3) of muscle fibers attached to tendon that were fixed in the same solution for an additional hour. After washing, samples were post-fixed with 1% osmium tetroxide for 1 h in the same buffer, rinsed in cacodylate buffer and dehydrated in a graded series of ethanols. They were embedded in epoxy resin and sectioned longitudinally (Law *et al.*, 1995). Semithin sections, stained with 1% toluidine blue in distilled water at 60°C, were observed by light microscope. The sections were trimmed to produce a thorough longitudinal plane of the muscle fibers, allowing clear MTJ identification. Thin sections, stained with uranyl acetate and lead citrate, were then observed with a Philips CM10 electron microscope (TEM) (Salucci *et al.*, 2013).

Morphometric analysis

Morphometric analysis was performed on the two groups of rats: CTRL and HS. Only the MTJ portions perpendicular to the main axis of skeletal muscle cells were evaluated (Figure 1A). The base (B) and perimeter length (P) of tendon finger-like processes at the MTJ were measured in semiautomatic mode, using the image analysis software ImageJ. The P/B ratio was taken as a measure of MTJ surface area and it was considered as an indicator of muscle tendon interface complexity (De Palma *et al.*, 2011). All the tendon protrusions longer than 0.2 µm were considered as primary finger-like processes. To know the extent of muscle-tendon interpenetration, in each MTJ portion, the finger-like processes extension (PL) was measured (Figure 1B).

All results were compared using Student's t-test. Significance was set at P≤0.01.

Results

Morphological and morphometric analysis showed interesting differences between the two groups. In fact, in CTRL rats, a lot of tendon interdigitations, penetrating the muscle mass, were visible and the interface profile appeared very folded (Figure 2A). On the other hand, in the HS group, a lower number of muscle-tendon interdigitations appeared. Finger-like processes were frequently absent and, where present, they appeared small and irregular (Figure 2B). In the CTRL group, the muscle ultrastructure revealed a normal sarcomere arrangement, with aligned myofilaments and the cytoplasm of muscle fibers appeared well-organized in thin areas between myofilaments. At high magnification, undamaged mitochondria and triads were observable (Figure 2C-D).

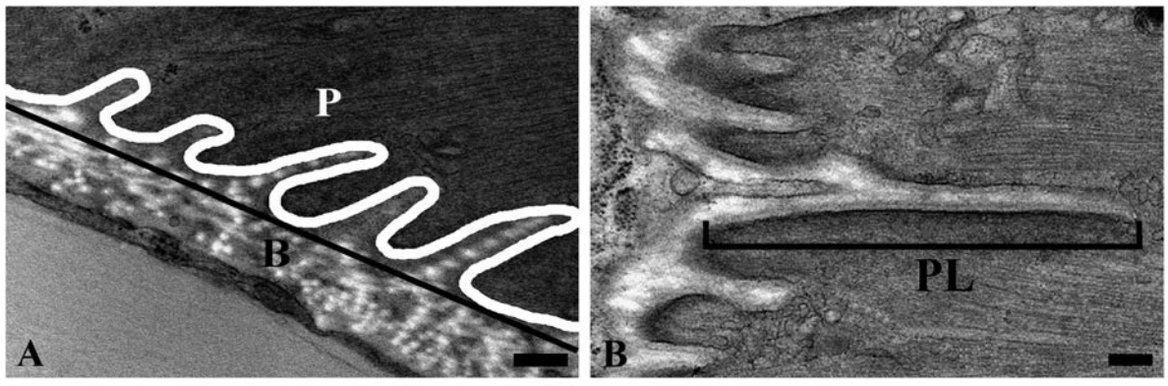

Figure 1. Morphometry of MTJs with *ImageJ* software. A) MTJ from CTRL group, perpendicular to the main axis of myofibrils, where the base (B) and perimeter (P) lengths of junction were highlighted . B) Primary finger-like process extension (PL) within the muscle. A, B Bar 0.25 µm

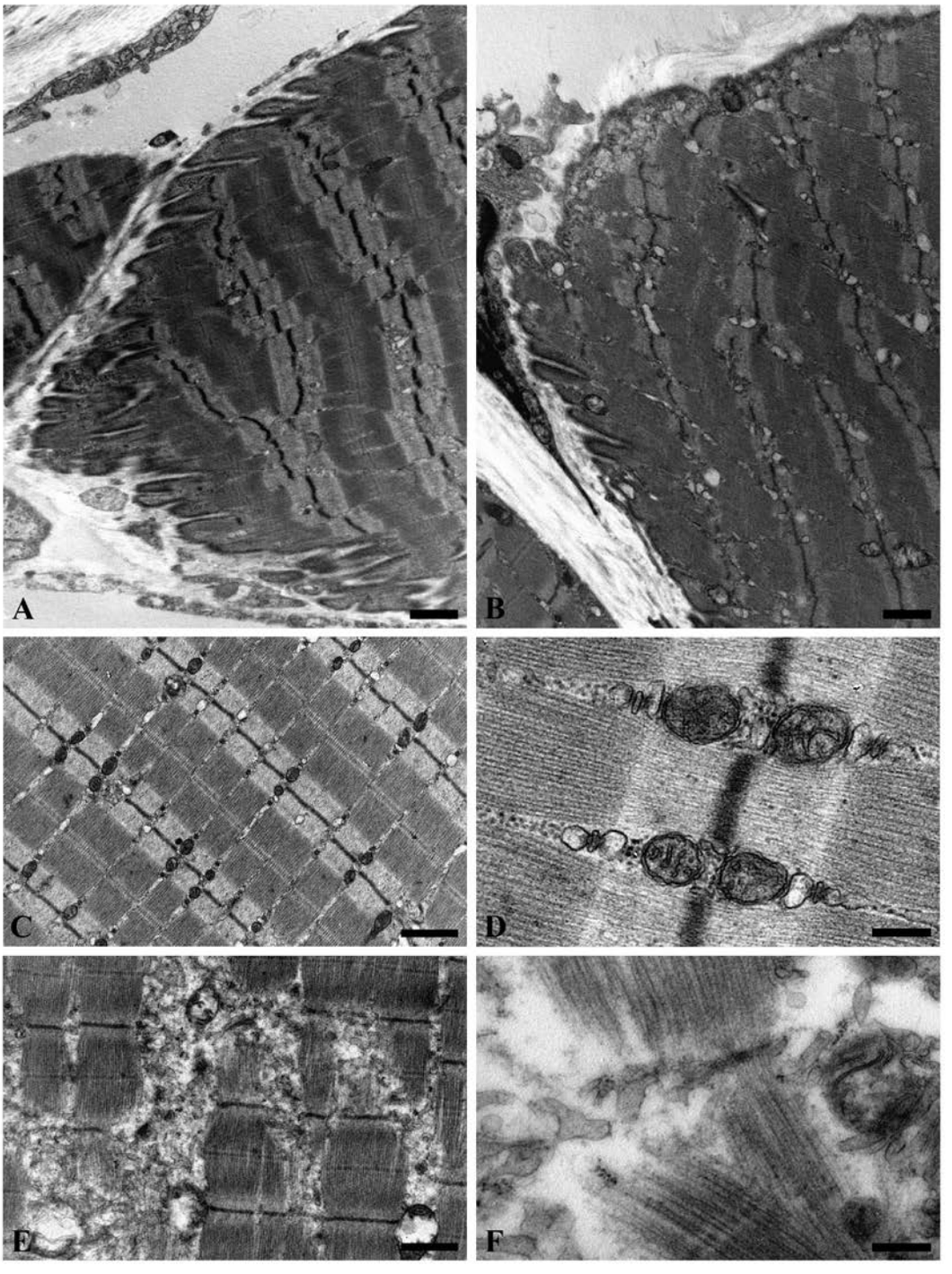

Figure 2. Plantaris MTJ, from CTRL (A-C-D) and HS rats (B-E-F). A) Long and several tendon interdigitations penetrate in to the muscle mass, parallel to myofilament orientation. B) After suspension, short and rare finger-like processes appear. C) Close to MTJ, the CTRL muscle ultrastructure appears well organized with aligned Z lines and thin intermyofibrillar cytoplasm areas. D) The cytoplasm shows a natural location of mitochondria and triads. E) In the HS rats, near the MTJ, misaligned sarcomeres are observed and swollen mitochondria and triads lose their natural location. F) At high magnification, disorganization of myofilaments and distortion or absence Z lines are observable. A, B Bar 1µm; C, E Bar 0.5µm; D, F Bar 0.25µm.

After 5 days of suspension, the skeletal muscle ultrastructure revealed signs of atrophy in response to decreased mechanical loading. In fact, close to MTJ, irregular and misaligned sarcomeres with absent Z lines, were observed. There was a certain loss of myofilaments, that often changed their orientation. The muscle intermyofibrillar area spread and glycogen granules occupied the degenerated regions. In muscle fiber cytoplasm, the sarcoplasmic reticulum was dilated and severely compromised. Mitochondria often appeared swollen and showed disrupted cristae. (Figure 2E-F).

Morphometric analysis were carried out in MTJ portions perpendicular to the main axis of muscle fibers in CTRL and HS rats. The hind-limb suspension induced significant MTJ modifications, with lower P/B ratio respect to the CTRL. In fact, as shown in Figure 3A, P/B ± s.e.m. decreased from 6.39±0.52 to 3.92±0.45, in CTRL and HS group respectively.

The decrease of P/B ratio indicates a reduced folding of muscle-tendon interface and, then, a lower surface area of MTJ. To understand how the surface area changed in HS animals, we measured the finger-like processes extension within the muscle (Figure 3B). This condition showed a significant decrease of finger-like process extension respect to CTRL rats, indicating a lower muscle-tendon interpenetration. In particular, the PL ± s.e.m. decreased from 1.84±0.21 to 0.97±0.09, in CTRL and HS group respectively.

Discussion

MTJs are specialized sites of muscle-tendon interactions and muscular force transmission from myofibrils to the collagen fibrils. The muscle fibre and the tendon interdigitate, which results in an amplification of the interfacial membrane area and in a reduction in local stress (force per unit area) (Kannus *et al.*, 1992). MTJs are also sites where forces are transmitted between myofibrils and the extracellular matrix and the region where muscle injury is predisposed during excessive strains during eccentric contractions (St. Pierre and Tidball, 1994). Several studies analyzed the muscle or tendon changes, generated by disuse, but we thought that it was necessary to study the relationship between muscle and tendon, because the modifications of single tissues could not explain the restoration of musculoskeletal system complexity. Usually the tendon tissue is partially protected from rapid changes in tissue mass, while muscle, which is known to act as a protein store for the organism (Heinemeier *et al.*, 2009), is subject to substantial and fast changes in tissue mass. However, it should be considered that important changes might have occurred in the tendon tissue despite the unchanged tissue mass.

Previous studies have provided evidence that MTJs undergo remodeling in response to changes in muscle rest length or loading (Wehling *et al.*, 2000; Chopard *et al.*, 2001). For example, during stretch-induced growth, sarcomeres are added in

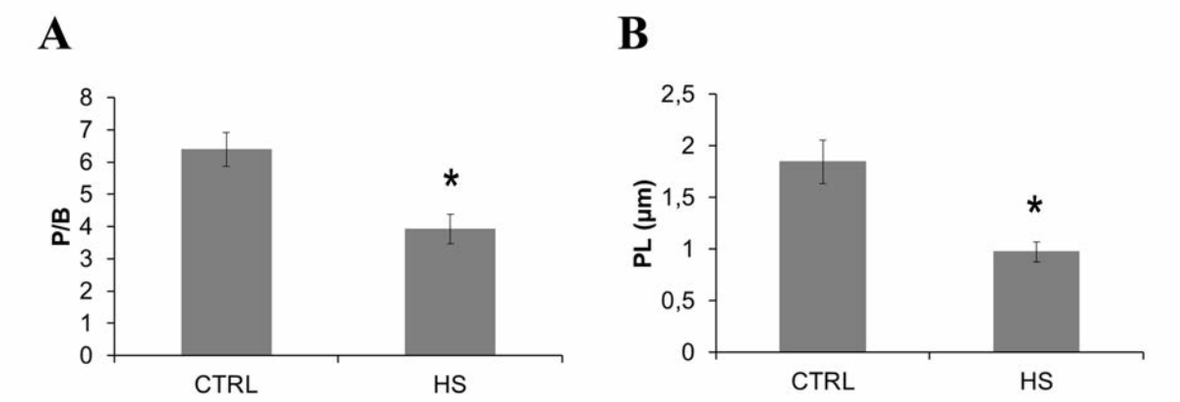

Figure 3. Results of morphometric analysis on muscle-tendon junction in CTRL and HS rats. A) Perimeter length/base length ratio (P/B), * P<0.01 by Student's t-test. B) Primary finger-like processes extension within muscle, * P<0.01 by Student's t-test. The histograms in A and B represent mean ± s.e.m.

series at fiber ends (making this model of rapid growth ideal for the study of myofibrillogenesis and mRNA localization). Also, stretching muscle leads to the accumulation of polysomes and mitochondria at the MTJ (Dix and Eisenberg *et al.*, 1990). Differently, a reduction in muscle loading associated with spaceflight results in a substantial decrease in force transmitting surface area at MTJ level as well as increases in ribosome and mitochondria concentrations at the MTJ. These changes in muscle structure and protein synthesis, that are restricted to MTJs in muscle, suggest that these aspects of muscle remodeling are regulated locally in the muscle fiber. We demonstrate that after only 5 days of suspension not only in the muscle but also at MTJ level ultrastructural modifications are evident. In response to unloading, we observe, by evaluating P/B ratio, a significantly reduction of the contact surface between muscle and tendon, as well as a decrease of tendon finger-like processes length. It is not well known whether unloading of tendon tissue could reduce the expression of collagen and collagen-inducing growth factors (Heinemeier *et al.*, 2009). Our results appear in agreement with the hypothesis that mechanical stress deprivation downregulate anabolic ECM pathways in tendon by reducing the expression of collagen I, collagen II, collagen III, aggrecan, decorin, and fibronectin. At the same time, it increases the catabolic process of the ECM by increasing the expression of matrix metalloproteinases (MMPs), especially MMP2 and MMP14, and reducing the expression of tissue inhibitor of metalloproteinase 1 (TIMP1) and TIMP2 (Sun *et al.* 2010). MMPs are a family of zinc dependent endopeptidases which collectively degrade essentially all the components of the extracellular matrix. These are involved in the tendon maintenance/remodelling, growth and development are highly dependent on a series of tightly regulated biological events that include matrix molecule extracellular proteolysis (Thornton *et al.*, 2010).

This work should be viewed as the starting point for more investigations. In particular, we plan to further analyze the possible ultrastructural effects of particular rehabilitation protocols on MTJ.

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