Increased expression of titin in mouse *gastrocnemius* muscle in response to an endurance-training program

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Titin, a sarcomeric giant protein, plays crucial roles in muscle assembly, elasticity and stability. Little is known about titin adaptation to endurance exercise. We studied the effects of endurance training on titin expression in mouse *gastrocnemius* muscles (MGM). Sixty-three ten-week-old male Swiss mice were divided into seven groups. Four groups were composed of untrained control animals (C0, C15, C30, C45) instead the other three included mice trained for 15 (T15), 30 (T30) and 45 (T45) days by treadmill. The training protocol was mainly aerobic, characterized by moderate-intensity, rhythmic and continuous exercises. Titin expression was determined by immunohistochemistry on MGM sections. Results revealed a significant reduction in body weight of the T45 mice and a significant increase in titin expression (% titin immunoreactivity median [range] = 41.11 [20-60] vs. 30.00 [10-50]). It is postulated that the up-regulation of titin expression is an adaptative mechanism to increase muscle elasticity and stability in response to the high number of stretch-shorten cycles during endurance training. Such a mechanism may be important for minimizing muscle energy consumption and improving performance during running.

Key words: titin, muscle elasticity, sarcomere, endurance training, expression, gastrocnemius, muscle.

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Despite the hereditary component involved in defining muscle type, a number of studies have shown that skeletal muscle can undergo different, significant morpho-functional adaptations dependent upon the intensity, duration and type of exercise training (Kyrolainen *et al.,* 2005; Matoba and Gollnick, 1984) due to the expression of certain genes induced by specific environmental stimuli (Wittwer *et al.,* 2004). It is well documented that muscle fiber type is tightly correlated to the expression of specific isoforms of myosin heavy chain (MyHC) (Campos, 2002; Gollnick and Matoba, 1984; Pette and Staron, 2000). The slow or fast fiber percentage in muscle can be associated with the features of the sport practised as shown by some reports (Matoba and Gollnick, 1984; Putman *et al.,* 2004; Ricoy *et al.*, 1998). For example, elite endurance athletes possess a higher percentage of slow twitch fibers in the muscle groups involved in the exercise (90-95% in gastrocnemius muscle) compared to untrained individuals; by contrast, elite sprinters and power athletes contain predominately fast fibers in the muscles that are used in their sport (Gollnick and Matoba, 1984).

Different types of muscles exhibit differences in force-extension behaviour, reflecting, on the one hand, variations in the content of extracellular and extrasarcomeric factors and, on the other, differences in the extensible properties of the endosarcomeric proteins (Gregorio *et al.*, 1999; Linke, 2000).

The filamentous intrasarcomeric protein titin, also called connectin, is one of the main determinants of muscle assembly, elasticity and stability (Wang *et al.,* 1979; 1984; 2001). Titin is a giant protein with a molecular weight ranging from 3.0 to 3.7 MDa depending on the isoform, and is more than 1 μm long (Nagy *et al.,* 2004; 2005). The molecule spans half the sarcomere, with its C and N termini, respectively, in the M and Z-lines, maintaining the central position of the thick filament in the sarcomere, which ensures balance of forces between its halves

during muscle contraction or passive extension (Tskhovrebova and Trinick, 2001).

In the A-band, titin is part of the thick filament and, both near and within the Z-line, it interacts with actin and Z-line proteins. In the I-band, titin forms a flexible connection between the end of the thick filament and the Z-disc. Mobility between domains is likely to be the major source of the flexibility in titin. Flexibility is especially important in the I-band region in which it changes its conformation, coiling up or elongating, and is the basis of elasticity in relaxed sarcomeres (Trinick, 1996). This region also reveals a large variation between isoforms (Freiburg *et al.,* 2000) correlated with the structure and elastic properties of the different muscle types, emphasizing the structural and functional role of the protein (Minajeva *et al.,* 2001). Titin provides a scaffold not only for most structural proteins contributing to the structural integrity of the sarcomere, but also for many cytoplasmatic proteins offering site-specific links with regulatory systems (Mayans *et al.,* 1999; Tskhovrebova and Trinick, 2004). Indeed, both ends of the molecule have potential phosphorylation sites that may be involved in protein signalling (Lange *et al.,* 2005).

Titin is usually regarded as a passive force generator in the sarcomere; however, recent lines of evidence have indicated that it is involved in the regulation of active tension generated by the actin-myosin complex (Nienderlander *et al.,* 2004). Few studies have focused their attention on the difference of titin quantities and isoforms in the skeletal muscle in response to exercise training (McGuigan *et al.,* 2003; Trappe *et al.,* 2002).

The goal of the present study was to examine the effects of an endurance training protocol on titin expression in the mouse gastrocnemius muscle.

Materials and Methods

Animal care

Experiments were carried out on 63, 10-week-old male Swiss mice that had an initial mean body weight of 38.00±3.87 g. The investigation conforms to the *Guide for the Care and Use of Laboratory Animals* (NIH Publication No. 85-23, revised 1996). All animals were familiarized with treadmill running for 1 week before beginning the exercise training. This preliminary exercise was limited to 5-10 min. Mice were randomly divided into 7 groups, each of these including 9 animals. Four groups were randomly selected as sedentary controls (C) (0, 15, 30 and 45 days). The three experimental groups were treated as follows: animals of group 1 (T15) were exercised for 15 days; group 2 (T30) for 30 days and group 3 (T45) for 45 days.

Table 1. Endurance training program.

Week No.	*Running speed (m/s)*	*Training time (min)*
1	0,053	15
2	0,053	30
3	0,066	30
4	0,066	44
5	0,066	60
6	0,08	60

Endurance training program

Mice were trained on a horizontally rotating treadmill and the program consisted of a training frequency of 5 days/week for 6 weeks increasing gradually both the workload intensity and the training time. The training protocol was predominately aerobic, characterized by moderate intensity, rhythmic and continuous exercises. Control mice did not undergo any training exercise. The data concerning the training speed and time for each week are shown in the Table 1.

In previous studies, we demonstrated that this training program induced cardiac and skeletal muscle hypertrophy in the mice, a criterion considered to be a good marker of endurance training efficiency (Di Felice *et al.,* 2007; Bellafiore *et al.*, 2007).

Immunohistochemistry analysis

In order to examine titin expression, the whole gastrocnemius muscle was excised from each mouse at the end of the training period (Di Felice *et al.,* 2007) and fixed with formalin. Portions of the muscle were embedded in paraffin and cut to obtain 5 μm sections which were used for the immunohistochemical analyses. After incubation for 10 min with 0.3% H_2O_2, serum-free protein block (DAKO, Carpinteria, USA) was added for 10 min. Sections were then incubated with the primary antibody against titin (Santa Cruz, California, USA) at a 1:20 dilution for 1 h at room temperature. Nonimmune mouse serum was substituted for negative controls. Troponin T antibody (Neomarkers, California, USA) was used as positive control and to normalize titin expression. After incubation for 10 min with biotinylated secondary antibody, AEC

chromogen (DAKO, Carpinteria, USA) was used to develop the HRP-streptavidin complex. The semi-quantitative expression of titin was assessed by two independent observers who were unaware of the experimental group from which the biopsy specimens were derived. Each observer quantified the immunoreactivity percentage for titin protein in 10 high-power fields (x40) for each slide. The date are expressed as the means of the values obtained.

Statistical analysis

The coefficient of variation of the immunoreactivity data noticed by the two observers was calculated, giving an indication of their variability. Means of the duplicate observations were analyzed using the Mann-Whitney U test and the medians of the groups were compared. A $p<0.05$ was considered significant.

Results

During the training period, there was a physiological weight increase due to normal growth. The T15 and T30 animals did not show any significant difference with respect to the C15 (40.17±3.70 g vs. 39.23±2.50 g) and C30 (46.00±6.63 g vs. 47.82±4.85 g) groups, respectively. After 45 days of endurance training, the mice showed a significant reduction in body weight (48.43±1.99 g vs. 44.17±7.13 g, $p<0.05$) in comparison with the corresponding controls.

The immunohistochemical analyses exhibited titin localized inside the muscle fibers (Figure 1). The expression of troponin T did not significantly differ between control and trained groups (*data not shown*). As the coefficient of variation in the data from the two observers was less than or equal to 0.5, the mean of the data from each group is a cor-

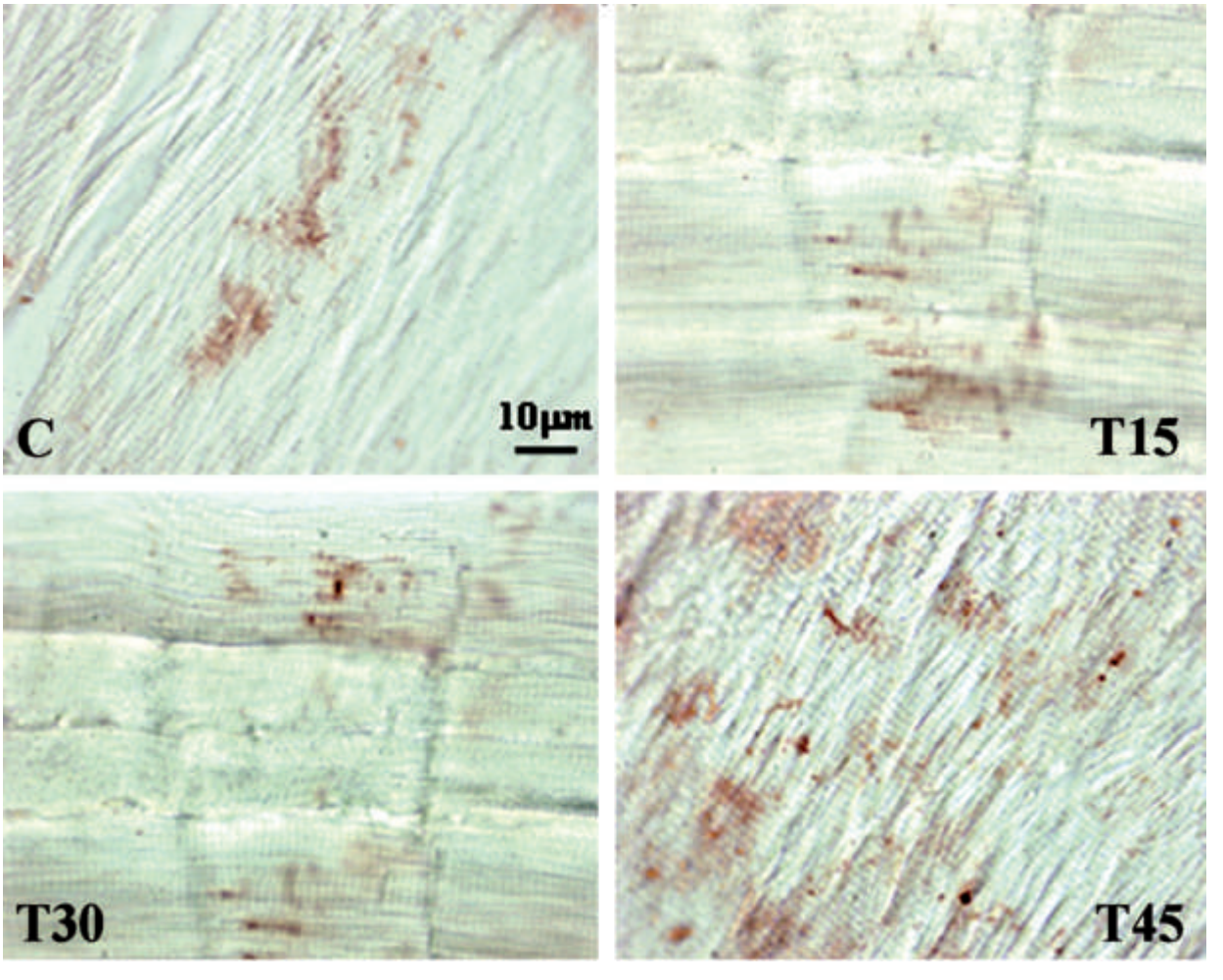

Figure 1. Representative microphotographs showing titin expression in the *gastrocnemius* muscle sections from trained and control mice by immunohistochemical analysis. There was no significant difference between control animal groups. The expression of titin significantly increased after 45 days of endurance training.

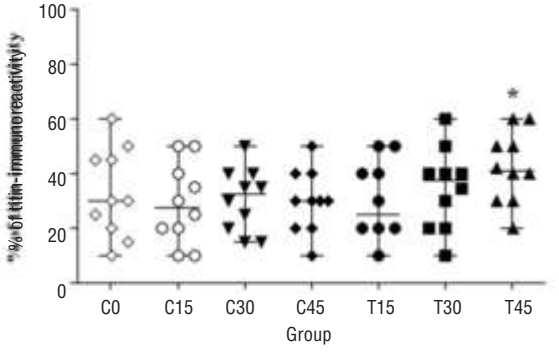

Figure 2. Statistic evaluation performed by the Mann-Whitney U test illustrates that percentage of titin immunoreactivity was significantly higher ($p<0.05$) in mice trained for 45 days than control animals. Titin expression was normalized to troponin T expression. *$p<0.05$ trained vs. control mice.

rect indicator statistically. This semi-quantitative evaluation indicated that titin expression was unchanged ($p>0.05$) in the *gastrocnemius* muscles of the control groups (Figure 2). Moreover, the mice in the T15 and T30 groups did not show any significant difference in titin expression ($p>0.05$) compared with control animals (% titin immunoreactivity median [range] = 25.00 [10-50] and 37.22 [10-60] vs. 30.00 [10-50]) (Figure 2). By contrast, levels of titin expression were significantly higher by 1.4 times in T45 animals (% titin immunoreactivity median [range] = 41.11 [20-60] vs. 30.00 [10-50]) than the control ones ($p=0.0262$). In addition, no significant differences between T15/T30 and T45 animals were noticed ($p=0.0753$, $p=0.1237$ respectively) (Figure 2).

Discussion

Size and allocation of titin in the sarcomere structure establish its importance in the mechanisms of muscle elasticity (Tskhovrebova and Trinick, 2000; Anderson *et al.,* 2002). This function is the result of the extensibility of the molecule: indeed, from a quantitative analysis of the flexibility and extensibility of isolated titin proteins, visualized by electron microscopy, a relationship between the conformation of titin and the magnitude of the strength applied was found (Tskhovrebova and Trinick, 2001).

The importance of titin integrity for normal muscular function has been shown both *in vivo* and *in vitro* in skeletal muscle dystrophies (Nienderlander *et al.*, 2004). The literature is inconsistent regarding changes in titin expression in response to exercise training of long duration and its involvement in sport performance. Mc Guigan *et al.* (2003) determined changes in titin and myosin MyHC isoforms in *vastus lateralis* muscle from twenty-four male subjects after explosive jump squat training for 8 weeks and showed that there was no significant difference in the expression of these two isoforms between trained and untrained subjects.

In a study carried out by Trappe *et al.* (2002), titin and nebulin content was measured in muscle biopsies from the *vastus lateralis* before and 24 h after a bout of high-intensity eccentric knee extensor resistance exercise in seven men. These authors observed that titin titin and nebulin amounts were significantly reduced ($p<0.05$) after exercise by 30 and 15%, respectively, suggesting that the structural components of the myofibrillar apparatus were degraded in this type of exercise in humans. These findings were also supported by Komi (2000), who reported that mechanical direct perturbations in the structural proteins of the sarcomere, such as titin, can occur after maximal and brief cyclical exercises.

Titin expression was also examined in different athletic populations (5 subjects for each group) with increased levels of strength and power (weightlifters, powerlifters, sprinters) compared with non-athletes. One-repetition maximum in the squat exercise and counter movement vertical jump trials were performed to assess strength and power capabilities, respectively. Gel electrophoresis analyses of muscle samples indicated that non-athlete groups presented lower titin-1 (intact titin) and higher titin-2 (degraded titin) percentages than the weightlifter, powerlifter and sprinter groups (McBride *et al.,* 2003). This investigation showed that there was a differential expression of titin protein bands in competitive athletes with increased levels of strength and power in comparison to untrained non-athletic individuals. However, it is not known if the two bands were isoforms or proteolytic fragments dependent on the exercise type, because molecular weight standards for titin did not yet exist. Some relationships between titin characteristics and athletic performance were observed; however, no conclusions have been drawn with respect to the contribution of titin to strength or power capabilities (McBride *et al.,* 2003).

The relationship between the modifications in titin molecular structure and neuromuscular performance was assessed on *gastrocnemius* muscle biopsies from 23 subjects who performed explosive

muscular exercises for 15 weeks. A significant increase in strength was observed, but titin and MyHC expression remained unchanged (Kyrolainen *et al.,* 2005).

In contrast with all these reports that focused their attention on the variation of titin expression in response to strength and power training, we examined titin expression induced by endurance training. In the present study, 45 days of exercise training but not 15 or 30 days resulted in a variation of titin expression. In particular, we observed a significant increase of titin in the mouse *gastrocnemius* muscle after 6 weeks of endurance training. The antibody and method that we used in these experiments could not discriminate between the different isoforms of titin; thus, the results describe the expression of all titin molecules. The lack of a reduction in titin content suggested that the endurance training protocol did not evoke long-term muscle damage. The intensity and modality of the workload in the endurance training could be stimuli that induce titin expression. In this respect, recent studies have identified a titin kinase domain-associated signalling complex which functions in response to mechanical stretch to regulate muscle gene transcription (Lange *et al.,* 2005). Titin upregulated expression has been also found in the *vastus lateralis* muscle of 16 subjects in response to 20 week endurance training, and appears to play an interesting role in the improvement of insulin sensitivity (Teran-Garcia *et al.,* 2005). However, the involved signalling pathways are unknown.

The increased expression of titin could be a structural adaptation of the muscles involved in running to maximize the storage and release of elastic recoil energy. In this respect, during running, mammals seem to select stride frequencies that maximize the muscle activity of alternate stretch-shorten patterns, and as a consequence, they reduce the expenditure of the energy for the locomotion (Taylor, 1985). Results from measurements on the long heads of rat *triceps brachii* muscle indicated that the group exercised by a chronic eccentric training produced significantly more passive and active lengthening force compared with sedentary animals (Reich *et al.*, 2000). Reich *et al.* (2000) suggested that the changes in the muscle elastic properties, for which titin may be responsible, may serve as a mechanism protecting the muscle from possible damage due to eccentric training. In addition, analysis of treadmill locomotion in the heterozygous B6-+/*mdm* mice, which present a mutation in the titin coding region, did not have any apparent muscle pathology, but showed an altered gait including stride, stance and swing time, as compared to B6-+/+ controls (Huebsch KA *et al.*, 2005). Therefore, titin also plays a functional role in the dynamics of muscle contraction.

To the best of our knowledge, the present work is the first that analyzes titin expression after endurance training in mice. Further studies are being carried out on different muscle types (fast and slow fibers) to quantify titin expression in response to different training protocols.

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