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Alterations in Myonuclear Number and BDNF Expression in Soleus Muscle Fibres Following Endurance Training are Androgen-Dependent

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The aim was to determine the changes in myonuclear number, cross-sectional area (CSA) and the expression of brain–derived neurotrophic factor (BDNF) in soleus muscle of endurance trained rats treated with androgen receptorblockers (ARB). Male Wistar rats were divided into 3 groups: non-trained (NT), trained (T) and trained receiving Flutamide (T+F). The two trained groups were exercised on a treadmill for 8 weeks. Hematoxylin-eosin, Azan staining and immunohistochemical reaction for BDNF were applied. The nuclear number per fibre on cross-sections of the T+F group was lower compared to T ($P<0.001$) and higher compared to NT ($P<0.05$). No significant effects of training and ARB treatment on CSA were found ($P>0.05$). Strongest BDNF immunoexpression in muscle fibres and myoblasts of soleus was detected in the T group. The results suggest that androgens are involved in the process of submaximal training adaptation in slow-twitch muscle fibres of male rats via AR.

Key words: soleus, cross-sectional area, BDNF, Flutamide, endurance training

Introduction

Adaptation of skeletal muscles to exercise training occurs by change in their function and structure. This response is fully dependent on the type of training applied [25]. For instance, in resistance training muscle mass and myofibre cross-sectional area (CSA) are mainly increased. In regular endurance training alterations also involve metabolic adaptation combined with increase in fatigue resistance. Adaptation mechanisms include interaction between a multitude of organs, tissues, cells, subcellular structures and various signalling pathways [10, 13].

Traditionally, aerobic training is not associated with muscular hypertrophy; however, evidence in the accessible literature is controversial. A number of studies have demonstrated

an increase in the myofibre cross section size [11]. The increase in muscle mass is due to an increased protein synthesis in the muscles [22] and the formation of new myofibres with the participation of satellite cells (SCs) [25]. Under the influence of various stimuli (in muscle injury or exercise training) SCs are activated and give rise to daughter myogenic cells. Following several proliferation stages these myogenic cells participate in the formation of new muscle fibres [19, 21]. These adaptation alterations are associated with an increase in myonuclear number [24]. The mechanisms of hypertrophy and SCs activation in the growing myofibres are modulated by endocrine anabolic factors and locally expressed auto/paracrine growth factors [5], sensitive to exercise training [16]. A number of neurotrophic factors play a role in the activation of quiescent SCs [15, 18, 23].

Neurotrophins in skeletal muscles act as potential regulators of growth, maintenance, function and regeneration of muscle fibres. These neurotrophic factors modulate myoblast and myofibre differentiation [4]. The expression of BDNF in skeletal muscles is associated with SCs differentiation and is influenced by exercise [18]. There is evidence of an increased BDNF expression in homogenates from soleus muscle after 5-day treadmill training at a rate of 20 $m \cdot min^{-1}$ [20]. The molecular mechanisms responsible for these processes during endurance training and the role of androgens in these processes are still unclear [8, 9].

The aim of the present study was to investigate the changes in myocyte cross section and myonuclear number as signs of muscular hypertrophy, as well as the BDNF expression in soleus muscle of endurance trained rats receiving an androgen receptor blocker (ARB).

Materials and Methods

Male adult Wistar rats (baseline weight 180-200 g) were divided into two main groups – a non-trained one (NT, n=6) and a trained one (T, n=12). The trained rats were exercised on an EXER-3R-Treadmill (Columbus Instruments, Columbus, OHIO, USA) under conditions of submaximal training (70-75% VO_{2max}) 5 days a week for 8 weeks. Training duration was gradually increased during the first week, reaching 40 min per day in the second week and this duration was maintained until the end of the experiment. Half of the trained rats were treated with 15 $mg \cdot kg^{-1}$. Flutamide dissolved in sesame oil and administered subcutaneously (T+F), whereas the other half of the trained (T) and non-trained (NT) rats were treated with sesame oil for the same period of time. The experimental protocol was approved by the Ethical Committee on Human and Animal Experimentation of the Medical University - Plovdiv, and the Commission for Ethical Treatment of Animals at the Bulgarian Food Safety Agency. The rats were reared and all experimental procedures were performed according to the recommendations of the European Commission for the protection and humane treatment of laboratory animals.

Two days following the last exercise training the rats were decapitated under anaesthesia with Thiopental 30 $mg.kg^{-1}$, after which the soleus muscles of the animals were severed, fixed in Bouin's solution for 24 hours at room temperature and embedded in paraffin. Immunohistochemical reaction was applied to the paraffin-embedded sections (5 μm). Primary BDNF antibody (Santa Cruz Biotechnology, USA) and ImmunoCruz ABC Staining System (Santa Cruz Biotechnology, USA) were used. Some of the sections were investigated by hematoxylin-eosin staining and the Azan method.

Using the DP-Soft specialized software (Olympus, Japan) installed on a Microphot microscope (Nikon, Japan), the intensity of BDNF expression in the soleus muscle was recorded, as well as the cross sectional myonuclear number (in a sample of 50 fibres per animal) and the CSA (μm^2). The cross sections were investigated using one and the same magnification (x200). Data were analysed with one-way ANOVA and in cases

of significance of the F-criterion, Tuckey or Games-Howell *post hoc* test was applied, depending on the homogeneity of dispersions. Difference at $P<0.05$ was accepted as statistically significant. The results were presented as a mean ± standard error of the mean (x±SEM).

Results

Largest nuclear number per fibre on cross-sections was observed in the animals from the T group (7.52±0.21). In the T+F group myonuclear number was lower as compared to T ($P<0.001$), but higher as compared to NT ($P<0.05$) (**Fig. 1; Fig. 2**).

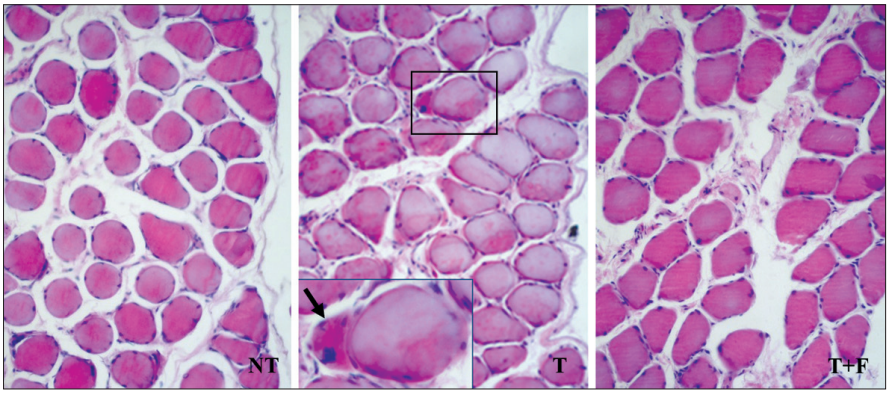

Fig. 1. H&E staining (*Magn. x 200*). Cross section of soleus muscle myofibres. **NT**. Non-trained control. **T**. Endurance-trained rats. Peripherally located myoblast (enlarged). **T+F**. Endurance-trained rats receiving ARB.

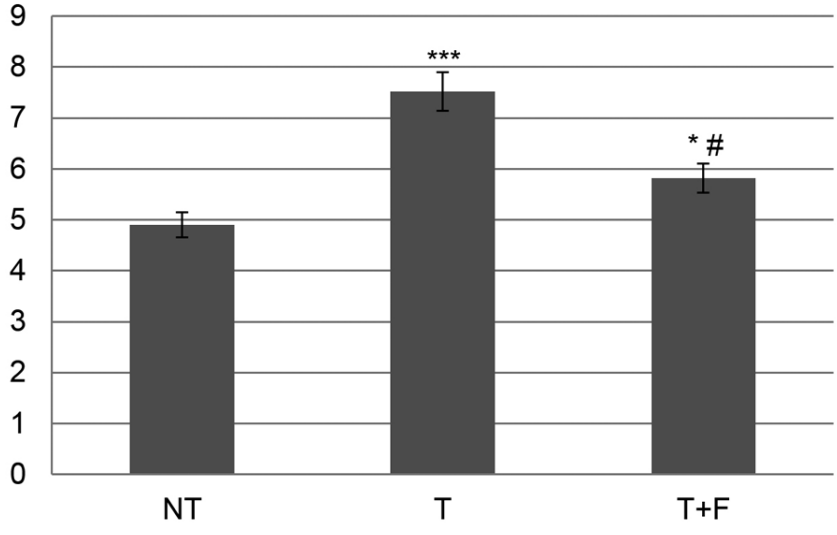

Fig. 2. Mean myonuclear number per fibre on cross section of soleus muscle. ***$P<0.001$ as compared to NT, *$P<0.05$ as compared to NT, $^{\#}P<0.001$ as compared to T.

The statistical analysis of the data obtained from the soleus muscle showed that trained rats had a larger cross section of the muscle fibres as compared to the non-trained controls ($P<0.05$). ARB administration reduced the CSA in the T+F group as compared to T, but a significant difference was not reached. As compared to the NT group, the CSA values in the T+F group were higher ($P<0.01$) (**Fig. 3; Fig. 4**).

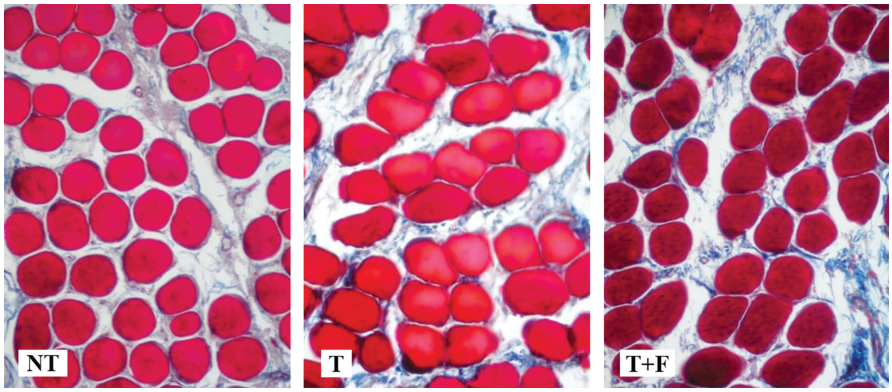

Fig. 3. Azan staining (*Magn. x 200*). Cross sections of soleus muscle of the animals from the experimental groups. **NT**. Non-trained control. **T**. Endurance-trained rats. **T+F**. Endurance-trained rats receiving ARB.

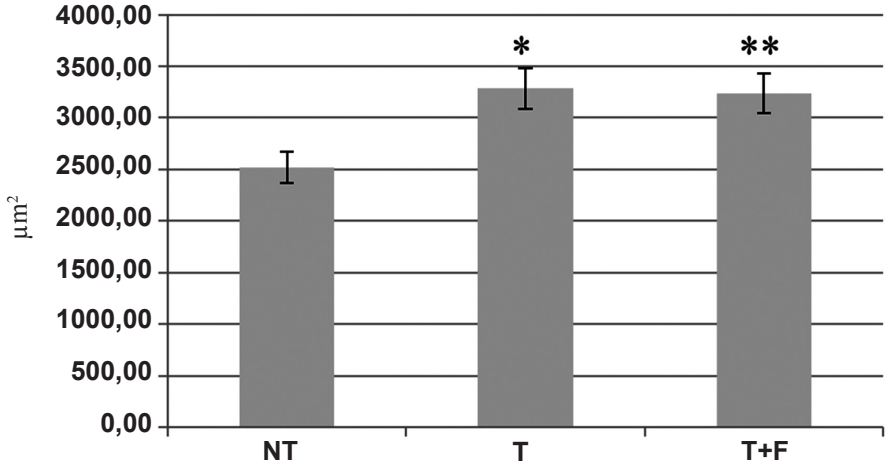

Fig. 4. Cross-sectional area (μm^2) of soleus muscle myofibres. *P<0.05, **P<0.01 as compared to NT

Immunoreactivity for BDNF in the soleus muscle fibres of the animals from the T group was significantly higher as compared to the NT group ($P=0.001$). Flutamide administration did not alter the intensity of the reaction for BDNF ($P>0.05$) in the muscle fibres.

Greatest intensity of immunoexpression was recorded in the newly formed myoblasts of the trained animals not receiving ARB, and the differences observed were significant not only in comparison to the non-trained animals (P<0.001), but also to the trained ones receiving ARB (P<0.01) (**Fig. 5; Fig. 6**).

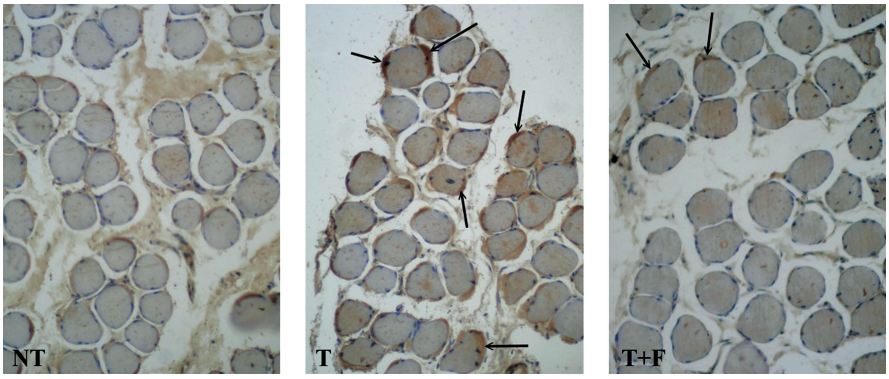

Fig. 5. BDNF immunoreactivity in soleus muscle on cross section (*Magn. x 200*). **NT**. Non-trained control. **T**. Endurance-trained rats. **T+F**. Endurance-trained rats receiving ARB. *Arrows* - expression in myoblasts located peripherally to myocytes.

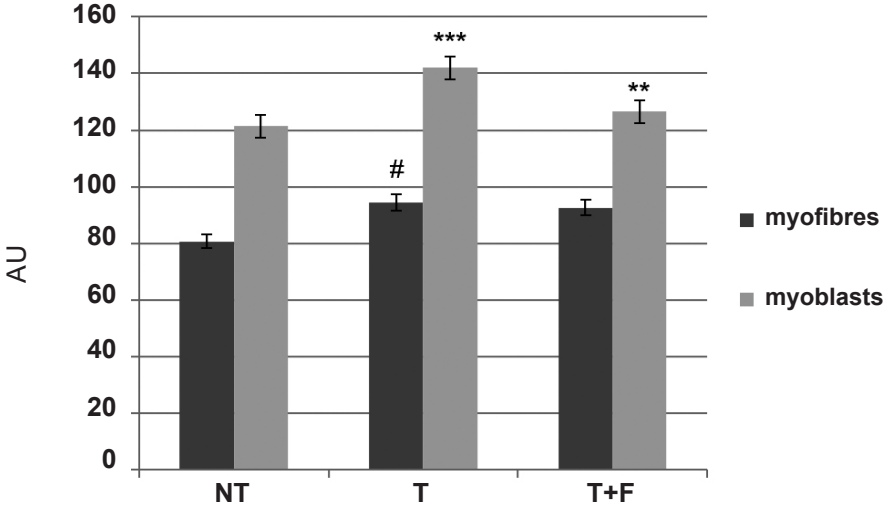

Fig. 6. Intensity of BDNF expression (AU) in myofibres of soleus muscle on cross section. #P<0.01 as compared to NT, ***P<0.001 as compared to NT, **P<0.01 as compared to T.

Discussion

Our results showed that endurance training increased the number of myonuclei and the myofibre cross-sectional area of soleus muscle. They are consistent with the results of other studies [7] that have also found a CSA increase in type I fibres of soleus and

EDL muscles of rats following a 10-week treadmill exercise under similar training conditions. In people, it has also been found that a 12-week aerobic training results in an increase in the mean cross section of type I and type II muscle fibres, accompanied by an increase in the number of SCs associated with type I fibres. A higher myonuclear number in type I fibres was observed as well [10].

These data support the hypothesis discussed lately concerning one of the mechanisms underlying the response of muscles to exercise training - formation of new myofibres [3, 21]. It has been found that in resistance training the increase in SCs is type-specific and occurs in type II myofibres, which are associated to a greatest degree with the development of muscular hypertrophy [26]. In aerobic training muscles made up of predominantly type I and type IIa fibres are involved, such as the soleus muscle (85% type I), [6]. We observed the largest myonuclear number per fibre on cross section in this muscle. Our results support the concept that new myonuclei are added to these muscle fibres, so that the latter can respond to the increased requirements resulting from regular aerobic training, expanding the so-called myonuclear domain [1]. This process occurs with the participation of stimulated SCs that proliferate and differentiate, since the nuclei in the mature myofibres are post-mitotic [24]. The so-formed myoblasts converge with the existing myofibres, providing nuclei as an additional source of protein synthesis.

The administration of ARB reduced the myonuclear number of soleus in the trained animals, as compared to the trained control. This fact can be explained with the absence of a testosterone (Ts) effect due to blocked androgen receptors (ARs). The latter are most likely localised in the muscle fibres and SCs [9, 14]. This alteration demonstrates the participation of androgens in the processes of adaptation of muscles made up mainly of type I fibres to aerobic training, an example of which is the soleus muscle.

The results obtained showed that regular submaximal training led to a significant increase in the CSA of soleus. Skeletal muscles are very plastic tissues capable of adapting to the increased motor and metabolic needs during training. A number of signalling pathways and myogenic regulatory factors are triggered, which activate protein synthesis (myofibrillar, sarcoplasmic, mitochondrial). These different types of protein synthesis in the muscles underlie the adaptation response to exercise training [2]. The enhanced protein production results in an increased myofibre bulk, which can be seen on cross section of the muscle. A number of studies have found an increase in myoglobin concentrations resulting from training or electric stimulation, which has been associated with the improved oxidative capacity of muscle fibres [17]. ARB administration in the trained animals reduced the myofibre CSA of soleus; however, the difference was not significant. These results can be explained with the different sensitivity of the fibre types to Ts and, to be more precise, of the ARs localised in them. Many authors share the opinion that type II myofibres are more sensitive to the action of Ts and its derivatives [12].

The type of exercise training determines to a greatest extent the presence of muscular hypertrophy [10]. This explains why some experimental models involving aerobic training have not found significant increase in myofibre bulk [13]. Prolonged training enhances proteolytic activity in the muscles mostly owing to the raised glucocorticoid levels. The exercise training applied in our experiment was submaximal, about 70-75% of VO_{2max}, which is more intensive than pure aerobic training (60-65% of VO_{2max}).

The increased BDNF expression that we found not only in the muscle fibres, but to a greater extent in the newly formed myoblasts of soleus, proves the participation of this

neurotrophin in the control and occurrence of part of the adaptation processes observed in skeletal muscles during training. McKay and colleagues report about cells of identical location (peripheral to the muscle fibres), which are BDNF-positive [18]. The authors emphasize the key role of BDNF in the late proliferation and early differentiation of SCs in vivo in humans.

AR blockade by Flutamide reduced BDNF expression in the myoblasts but did not influence its expression in the muscle fibres, which shows that androgens influence the synthesis of this neurotrophin mainly in the newly formed myofibres during training. Having auto/paracrine effects, BDNF is likely to participate in the mechanisms through which new muscle fibres are formed under conditions of submaximal training.

In conclusion, our results show that androgens participate in the adaptation to endurance training by means of their own ARs. This genomic mechanism is likely to be the one through which the increase in myonuclear number is influenced, as well as BDNF expression in the myofibres and the newly formed myoblasts of skeletal muscles made up mainly of type I fibres, an example of which is the soleus muscle.

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