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Green Tea Extract Increases the Expression of Genes Responsible for Regulation of Calcium Balance in Rat Slow-Twitch Muscles under Conditions of Exhausting Exercise

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We studied the role of calcium-regulating structures of slow- (*m. soleus*, SOL) and fast-twitch (*m. extensor digitorum longus*, EDL) skeletal muscles of rats during adaptation to exhausting physical activity and the possibility of modulating this adaptation with decaffeinated green tea extract. It was established that EDL adaptation is mainly aimed at Ca^{2+} elimination from the sarcoplasm by Ca-ATPase and its retention in the reticulum by calsequestrin. Administration of green tea extract increased endurance due to involvement of slow-twitch muscles whose adaptation is associated with enhanced expression of all the studied genes responsible for the regulation of Ca^{2+} balance.

Key Words: *m. soleus; m. extensor digitorum longus; calcium; exhausting exercise*

Analysis of mechanisms underlying muscle fatigue under conditions of intensive physical exercise and subsequent muscle function recovery is an important task of functional biochemistry and physiology. Fatigue at physical exercise is defined as a decrease in body's ability to maintain the required productivity level [6]. The biological meaning of fatigue is obvious and consists in limiting harmful consequences of exhausting work for the body. At present, there is no unified theory of fatigue of muscles working under conditions of a whole organism. Studies of molecular and cellular mechanisms of electromechanical coupling (EMC) gave rise to a concept of significant role of subcellular structures of muscle fibers (T-tubes, mitochondria, and sarcoplasmic reticulum myofibrils), posttranslational receptor modifications, ion channels and regulatory proteins in fatigue development [3,4,9]. The search for drugs of natural origin that reduce fatigue and increase physical endurance is a promising research trend in sports physiology and medicine [7]. The effectiveness of certain drugs and mechanisms underlying the influence of intensive exercise on muscle function are studied using specially designed training techniques followed by the analysis of a wide spectrum of functional, biochemical, and morphometric characteristics of the muscle [1,2]. Nutraceuticals (bioactive additives and food ingredients, minerals and metabolites of natural origin not included in the list of prohibited drugs) can increase physical tolerance and performance by improving the balance of signalling and metabolic processes in cells and tissues. The effect of many nutraceuticals, including decaffeinated green tea extract (GTE), is related to activation of lipid catabolism and ROS neutralization. However, the effect of GTE on the structure of EMC and protein regulators of Ca^{2+} balance in slow- and fast-twitch muscles in case of prolonged physical exercise has never been studied.

Here we studied the expression of genes responsible for calcium balance in rat fast- and slow-twitch skeletal muscles after forced swimming cycle against the background of GTE application.

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MATERIALS AND METHODS

The experiments were carried out in accordance with the Rules for Conducting Work on Experimental Animals approved by the Ethics Committee of I. M. Sechenov Institute of Evolutionary Physiology and Biochemistry. Male Wistar rats were kept under standard vivarium conditions; males weighing 200±10 g were preliminarily adapted to water for 5 days (water temperature of 32°C). On day 6, forced swimming testing was carried out that consisted of 3-min swimming with a load constituting 7% body weight with 1-min intervals at water temperature of 28°C until complete exhaustion.

Based on the result of testing, rats were randomized and divided into 3 groups (6 animals per group): intact animals (negative control), rats subjected to forced swimming and receiving water orally (positive control; "water" group), and rats subjected to forced swimming and receiving GTE (Sunphenon 90D; Taiyo International Inc.) dissolved in water, daily 2 h prior to training and 2 h after the end of training, orally in a dose of 12 mg/kg (by catechins); daily dose 24 mg/kg (GTE group). The animals were subjected to forced swimming for 6 days (rest on day 7). During 6 days of experimental week 2, the rats swam with a load of 7% of body weight: 3-min swimming trials with 1-min intervals (water temperature 28°C), total swimming duration ~50% of testing values. On day 1 of experimental week 3, intermediate testing was performed (3-min swimming with 1-min intervals at water temperature of 28°C until complete exhaustion), during the next 5 days, the rats were subjected to swimming lasting ~50% of intermediate testing time. During 4 days of week 4, the rats were subjected to daily swimming with a load of 7% body weight for 3 min with 1-min intervals at water temperature of 28°C until complete exhaustion (submersion for 10 sec). On day 4, blood samples were taken from the caudal vein in 5 min after the end of exercise for measurement of lactate concentration (biochemical control of exercise intensity). Lactate was measured in the whole blood by photometric method using portable Accutrend Plus biochemical analyzer (Roche Diagnostics GmbH). The data are presented as median (min; max). On day 5 of experimental week 4, *i.e.* 24 h after the end of exercise, the animals were decapitated, *m. soleus* (SOL) and *m. extensor digitorum longus* (EDL) were isolated, immediately frozen in liquid nitrogen, and stored at -70°C.

Cytoplasmic RNA was isolated from muscles by thermal phenol fractionation. Purity and concentration of the isolated RNA was determined spectrophotometrically on a BioSpec-nano (Shimadzu) spectrophotometer. DNA was removed by hydrolysis with RNAse-free DNase I (Thermo Scientific). Reverse transcription of mRNA (2 μg) was performed using OT-1 kit (Synthol) and oligo $(dT)_{15}$ primers according to manufacturer's recommendations. To normalize the results of real-time PCR after reverse transcription (RT-cPCR) a reference gene was selected from the list of constitutively expressed genes used previously in similar studies: genes of cyclophilin A (*CypA*), β-actin (*ActB*), phosphoglycerate kinase 1 (*Pgk-1*), hypoxanthine-guanine phosphoribosyltransferase (*HPRT*), ribosomal protein 13A (*Rpl13A*), activator protein of tyrosine-3-monooxygenase/tryptophan-5-monooxygenase (*YWHAZ*), TATA-binding protein (*Tbp*). Primers were selected using nucleotide NCBI BLAST sequence database taking into account the exon-intron structure to exclude amplification of genomic DNA. To determine the expression of candidate reference genes, mRNA preparations from 3 rats from each experimental group were used. Stability of expression of candidate reference genes was evaluated by processing RT-cPCR results with geNorm, Normfinder, BestKeeper, and comparative ΔΔCt algorithms. Final processing and ranking of the results was carried out using Reffinder software. *Rpl13A* gene demonstrating the highest expression stability under experimental conditions was used as the reference gene. The target genes were *CASQ1* (calsequestrin of skeletal muscles of fast-twitch muscle fibers), *SERCA1* (Ca-ATPase of the sarcoplasmic reticulum of fast-twitch muscle fibers), *SERCA2* (Ca-ATPase of the sarcoplasmic reticulum of slow-twitch muscle fibers), *RYR1* (ryanodine receptor of skeletal muscles) and *CACNA1S* (α1-subunit of the dihydropyridine receptor). RT-cPCR results were analyzed using REST-2009 software [13]. The number of repetitions for bootstrapping was 2000, which ensures estimation of statistical significance of the detected changes. The critical significance level (p) for testing statistical hypotheses was taken as 0.05, 0.01, or 0.001.

RESULTS

GTE increased swimming duration on days 1, 2 and 3 of maximum exercise intensity, as well as mean swimming duration, but the differences from the positive control were statistically insignificant. The mean swimming duration in the control group during the last 4 days of maximum exercise intensity was 9.5 min, the mean lactate level was 13 (7.8, 16.8) mmol/liter 5 min after the end of the exercise and returned to 4.3 (3.1, 5.2) mmol/liter after 1 h. The mean swimming duration in the GTE group on the last day of maximum exercise was 8.5 min, the mean lactate level was 11.3 (5.2, 17.8) mmol/liter in 5 min after the end of the exercise and 4.1 (2.4; 8.2) mmol/liter in 1 h. The

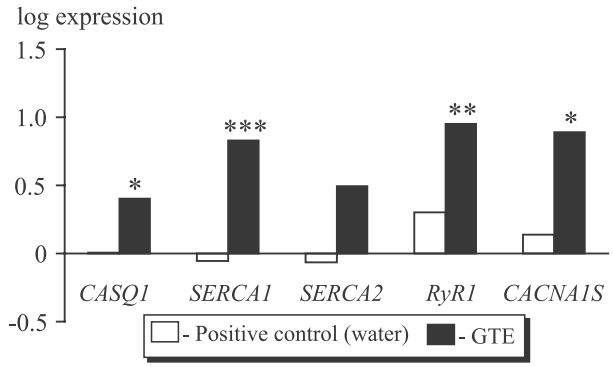

Fig. 1. Changes in the expression of genes of the calcium regulation system in rat SOL muscles after a cycle of physical activity and GTE administration. Here and in Fig. 2: *$p<0.05$, **$p<0.01$, ***$p<0.001$ in comparison with positive control.

3-fold increase in lactate level after exercise confirms its high intensity and adequacy of the applied model.

In SOL muscles of the positive control group, RT-cPCR did not reveal significant changes in the expression of the studied genes responsible for Ca^{2+} homeostasis (Fig. 1). At the same time, a statistically significant increase in the expression of *CASQ1* and *SERCA1* genes (by 3 and 11.5 times, respectively) was revealed in EDL muscles (Fig. 2). Expression of the following genes in rats receiving GTE and demonstrating higher performance was significantly elevated in not only EDL, but also SOL: *CASQ1* genes (by 2.5 times in both muscles), *SERCA1* (by 6.7 and 7.6 times, respectively), *SERCA2* genes (by 3 and 4 times), *RyR1* (by 9 and 4.7 times), and *CACNA1* (by 7.8 and 5.5 times). These findings suggest that adaptation of EDL muscles in the forced swimming model was mainly mediated by mechanisms of Ca^{2+} excretion from the sarcoplasm by Ca-ATPase and its retention in the sarcoplasmic reticulum with calsequestrin. Dihydropyridine and ryanodine channels play a secondary (auxiliary) role in the adaptation mechanisms. Attention should be drawn to the strong (4-fold), but statistically insignificant (due to the scatter of values) increase in *CACNA1* expression in EDL muscles of the positive control rats. It is obvious that white muscles "try" to establish coupling between the dihydropyridine and ryanodine receptors, *i.e.* to restore control in the "excitation—contraction" system (depolarization-induced calcium release, DICR) and thereby reduce the proportion of poorly controlled calcium-induced calcium release (CICR) through the ryanodine receptors. The number of free ryanodine receptors reaches 50% and they can serve as the main source of calcium overload under conditions of tetanic contraction.

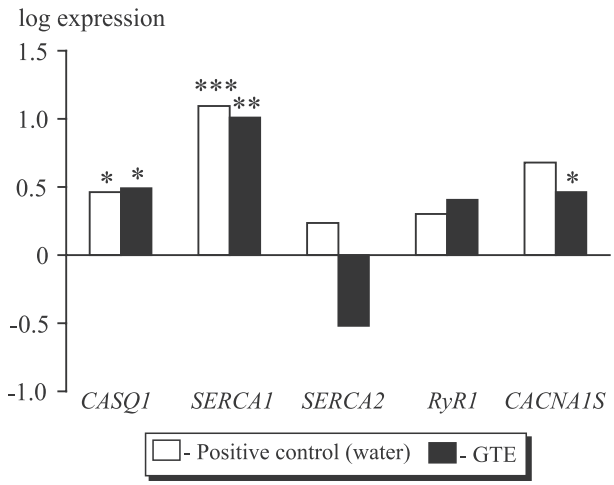

Fig. 2. Changes in the expression of genes of the calcium regulation system in rat EDL muscles after a cycle of physical activity and GTE administration.

Administration of GTE enhances endurance due to additional involvement of slow-twitch muscles, adaptation of which is associated with enhanced expression of all the studied genes responsible for Ca^{2+} balance regulation. Obviously, this kind of "transformation" can be explained by increased proportion of type 2A muscle fibers in SOL muscles, while the significant 3-fold increase in *SERCA2* expression in EDL muscles is indicative of increased proportion of type 1 muscle fibers. To verify these assumptions, it is necessary to investigate the expression of myosin heavy chain genes. Significant increase in *RyR1* and *CACNA1* expression, not only in fast-twitch but also in slow-twitch muscles in case of GTE application is indicative of effective EMC structures development in both types of muscles. Thus, in contrast to the "emergency" adaptation of muscles to exercise by means of calcium excretion from the cytosol and its retention in the reticulum of EDL (and probably in the mitochondria of SOL), adaptation aided by GTE is associated with increased control over calcium mobilization due to increased coupling in the "excitation—contraction" and "excitation—metabolism" systems.

The physiological and biochemical mechanisms of action of green tea catechins are of unquestionable interest for sports physiology and medicine, however, the studies are often separated, since functional and molecular effects of nutraceuticals are usually considered separately from the effects of the physical exercise itself. Earlier, the effectiveness of GTE was demonstrated in cases of chronic fatigue syndrome [15] and muscular dystrophy of Duchenne [5], immunomodulating properties of GTE under conditions of intensive physical exercise were established [12]. On the other hand, it was found that intensive interval exercise causes RyR1 fragmentation in skeletal muscles of untrained subjects due to Ca^{2+} leakage, increased ROS and activity of calpaines; in trained athletes this effect was not observed [14]. Western blotting showed an increase in *CASQ1* level in EDL muscles and its

reduced or unchanged level in SOL muscles of mice subjected to running exercise of both endurance and strength (sprint) types [11]. These data are consistent with the results of our studies and are indicative of adaptive mechanisms for Ca^{2+} level regulation, but it is important to understand how green tea catechins affect different stages of calcium balance regulation in slow- and fast-twitch muscle fibers under intensive loads in animals adapted to physical exercise. It is known that the short-term effect of green tea catechins is associated with catechol-O-methyltransferase inhibition, which, in synergy with caffeine, leads to an increase in catecholamine and cAMP level, enhances lipolysis and thermogenesis. Long-term effects of catechins are mediated by signal regulators PGC-1α (PPAR-γ coactivator 1-α) and PPARα, β/δ, γ; they are associated with increased expression of genes, responsible for fatty acid utilization [8]. In addition, catechins inhibit UDP-glucuronosyltransferase, indirectly regulating testosterone level [10]. We found that under conditions of experimental forced swimming including periods of adaptation as well as graded and maximum exercise intensity, adaptation of fast-twitch EDL muscles is associated with Ca^{2+} excretion from the sarcoplasm by Ca-ATPase and its retention in the reticulum by calsequestrin. In addition, we demonstrated that GTE increases endurance by means of additional involvement of slow-twitch muscles, adaptation of which is associated with increased expression of all the studied genes responsible for Ca^{2+} balance regulation.

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