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RESEARCH ARTICLE

Age-related peculiarities of change in content of free radical oxidation products in muscle during stress

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Abstract **The age-dependent peculiarities of stimulation of free radical processes in subcellular fractions of skeletal muscle of rats subjected to long-term immobilization stress were studied in order to improve knowledge about changes of muscular tissue during ontogenesis. It is found that adult animals do not show accumulation of proteins carbonyls, TBA-reactive substances, and Schiff bases in subcellular fractions of the thigh muscle when immobilized. Long-term immobilization causes apparent manifestation of oxidative stress only in mitochondrial fraction in pubertal rats. Mitochondrial oxidative stress defense systems are sufficiently effective, however, direction of pathways of free radical oxidation carbonyl products catabolism alters in the cytoplasm of myocytes in old rats under long-term immobilization conditions.**

Keywords ontogenesis, stress, skeletal muscle, free radical oxidation

Introduction

Significant changes accompanied by modulation of the strength of contraction take place in muscular tissue during ontogenesis. These changes are manifested by sarcopenia development with aging (Czarkowska-Paczek, Milczarczyk, 2006; Narici, Maffulli, 2010). Despite the widespread of this state (Rossi et al., 2008), the mechanisms of its formation are not clear. Nowadays it is known about the important role in this process of hormonal regulation disturbance of muscular tissue metabolism, change in response of muscle fibers to anabolic steroids and mitochondrial dysfunction in myocytes (Boirie, 2009; Frontera et al., 2012). Oxidative stress plays a special role in the development of sarcopenia (Rossi et al., 2008; Jackson, 2009; Calvani et al., 2013). However, data about the manifestation of oxidative stress in skeletal muscles during aging are controversial (Chen et al., 2008; Hindle et al., 2010). Muscular tissue response to pro-oxidant factors even less studied, although it is known that one of those – long-term immobilization, contributes to the development of this state (Chen et al., 2008). Taking into account the role of stress in the stimulation of free radical processes in the organism (Meerson, 1984; Davydov and Shvets, 2003), this study was undertaken to study age-related features of stimulation of free radical processes in the skeletal muscle in rats under long-term immobilization stress.

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Materials and methods

50 male Wistar rats of three different age groups were employed in the present study: 1 – 1.5-month-old (pubertal); 2 – 12-month-old (adult); 3 – 24-month-old (old) rats. Each age group was divided into two subgroups: 1 – intact ones and 2 – those affected by immobilization stress. Animals were immobilized by tying to a stationary plank for 5 h per day for 2 days. The effectiveness of stress was controlled by increasing level of 11-oxycorticosteroids and epinephrine in the blood of immobilized rats.

Fractionation of mitochondria from muscle is based on method given by Madsen et al. (1996) with some modifications. Rats were decapitated, then femoral muscle was removed and immediately placed in chilled 0.9% sodium chloride solution. Pieces of muscle tissue were thoroughly minced with scissors after washing from blood. Mince was

mixed at a ratio of 1:3 (weight: volume) with saline medium containing 0.05 M Tris, 0.005 M magnesium sulfate, and 0.001 M EDTA (pH 7.4) and then homogenized for 3 min in glass Potter-Elvehejm homogenizer with PTFE pestle. The homogenate was filtered through 4 layers of gauze and centrifuged at 1000 g for 10 min. The resulting supernatant was transferred to clean tubes and centrifuged at 10000 g for 20 min. The supernatant was used as postmitochondrial fraction. The precipitate was washed twice in homogenization medium at 10000 g for 20 min and used as a mitochondrial fraction. All fractionation procedures of muscle homogenate were performed at 4 – 5°C.

Content of substances which positively react with 2-thiobarbituric acid (TBA-reactive substances) (Esterbauer and Zollner, 1989), protein carbonyls (PC) (Fagan et al., 1999), as well as fluorescent products of metabolism of the Schiff bases type (SB) were determined in mitochondrial and postmitochondrial fractions of thigh muscle. Measurement of fluorescence was performed at 360 nm excitation wavelength and 430 nm emission wavelengths. Protein content in samples was determined by the Lowry method.

Statistical calculations were done by Wilcoxon-Mann-Witney test. Differences between the data were considered significant at $p < 0.05$.

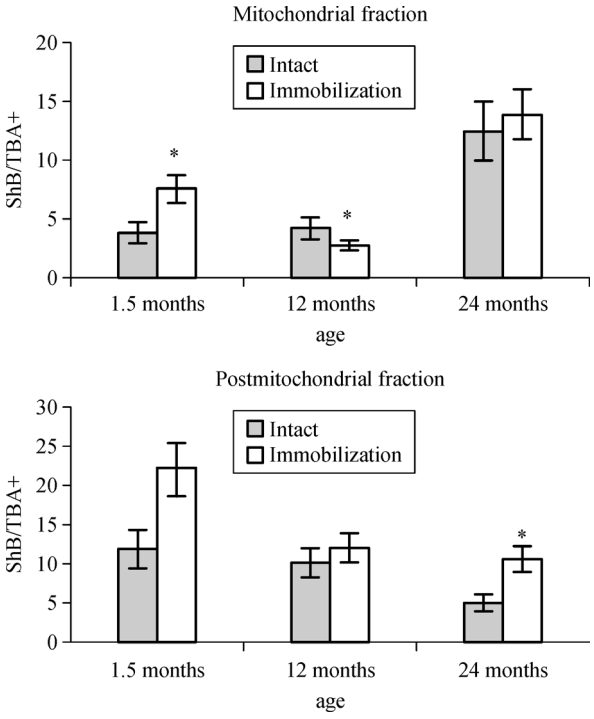

Figure 1 Ratio Shiff bases/TBA reactive substances (ShB/TBA+) in mitochondrial and postmitochondrial fraction of femoral muscle during stress (mean±SEM). The figure represents the average data from 6 investigations. * The data are positively distinguished from intact rats ($p < 0.05$).

Results and discussion

Studies showed increased content of PC and SB for 74% and 94% respectively as compared to initial levels in mitochondrial fractions of thigh muscle in 1.5-month-old rats after immobilization (Table 1). At the same time, the concentration of TBA-reactive substances remained unchanged. 12-Month-old rats showed 35% decrease in concentration of SB in the mitochondrial fraction as compared with its initial level after long-term immobilization. Content of TBA-reactive substances in this age group was not affected. Changes in content of free radical oxidation products of lipids and proteins in the mitochondrial fraction of the thigh muscle in 1.5-month-old rats were accompanied by parallel increase of SB/TBA+ index by 97% as compared to its initial level (Fig. 1). At the same time, 12-month-old animals showed 35% decrease of this value as compared with initial one. 24-Month-old animals did not show any significant change in the content of free radical oxidation products in the mitochondrial fraction of the thigh muscle after immobilization.

Thus, long-term immobilization of pubertal rats is accompanied by accumulation of free radical oxidation products of lipids and proteins, as well as by increase of SB/TBA+ index in the mitochondrial fraction of skeletal muscle, which can be considered as a manifestation of oxidative stress in the mitochondria of myocytes (Gianni et al., 2004). Moreover, oxidative stress in this age group is accompanied by change in utilization pathways of free radical oxidation carbonyl products. This is indicated by increased SB/TBA+ index in mitochondria in pubertal animals, which reflects the decreasing rate of catabolism of endogenous

Table 1 Level products of free radical oxidation in mitochondrial fraction of femoral muscle during stress (mean±SEM)

Age (months)	Experimental group of rats	Protein carbonyls (μmol/mg protein)	TBA+ substances (μmol/mg protein)	Shiff Bases (nmol/mg protein)
1.5	Intact	0.77±0.12	0.16±0.03	0.49±0.06
	Immobilized	1.34±0.02**	0.15±0.03	0.95±0.04**
12	Intact	1.90±0.27*	0.23±0.02*	0.94±0.03*
	Immobilized	1.39±0.29	0.24±0.03	0.59±0.06**
24	Intact	1.20±0.39	0.09±0.01	1.08±0.05*
	Immobilized	0.56±0.08	0.07±0.01	1.08±0.14

* The data are positively distinguished from intact1.5-months-old rats ($p < 0.05$).
** The data are positively distinguished from intact rats of same age group ($p < 0.05$).
The table represents the average data from 6 investigations.

aldehydes in enzymatic processes (Davydov et al., 2004). Probably, a significant portion of endogenous aldehydes enters non-enzymatic reactions resulting in formation of adducts with proteins (protein carbonyls and fluorescent products of metabolism of the Schiff bases type). There is no doubt that such changes contribute to stress damage of myocytes.

Adult and old animals did not show accumulation of free radical oxidation products of lipids and proteins in the mitochondrial fraction of the muscular tissue after long-term immobilization. Moreover, reducing of SB level was observed in adult rats. The reasons for this shift are not clear yet. Probably, these phenomena can be explained by increase of efficiency of antioxidant enzymes and intracellular repair systems, as well as by age-dependent phospholipid organization of mitochondrial membranes or by increased rate of degradation of intracellular proteins exposed to carbonylation or free radical oxidation.

Comparative evaluation of data obtained allows us to conclude that the mitochondria from pubertal rats' femoral muscle are the most sensitive to pro-oxidant effect of stress. Mitochondria from muscle cells of adult and old animals are highly resistant to pro-oxidant effects caused by long-term immobilization. However, adult rats show more pronounced adaptive changes in metabolism aimed for removing of covalently modified protein molecules.

Long-term immobilization did not cause changes in the content of PC in postmitochondrial fraction of thigh muscle in animals of all studied age groups (Table 2). At the same time, 12-month-old rats after immobilization showed 63% decrease in concentration of TBA-reactive substances in the given subcellular fraction as compared to the initial level. But in 1.5- and 24-month-old animals the TBA-reactive substances content remained unchanged. Level of SB in 12-month-old rats after immobilization was reduced by 24% as compared to the initial level. Old animals showed similar changes in SB level in postmitochondrial fraction but only tendentiously. However, a twofold increase of SB/TBA+ index was observed in old rats (Fig. 1). 1.5-Month-old rats showed tendency to increase the level of SB in postmitochondrial fraction after long-term immobilization. However, this change was not statistically significant ($p > 0.05$).

Thus, in postmitochondrial fraction of femoral muscle in 12-month old rats after immobilization, unlike 1.5- and 24-month-old animals, the level of free radical oxidation products of lipids (SB and TBA-reactive substances) reduces. This alteration may be caused by effective age-specific functioning of enzymatic antioxidant defense systems in the cytosol of myocytes as well as systems for utilization of carbonyl metabolic products and decomposition of covalently modified protein molecules.

Undoubtedly, metabolic systems of muscles anti-stress defense are also activated in old animals after immobilization. However, in contrast to adult rats, old ones show simultaneous changes in direction of pathways of catabolism of cytotoxic free radical oxidation carbonyl products in the cytoplasm of skeletal muscles cells. Judging by the nature of change of SB/TBA+ index, aldehydes are intensively used for the formation of adducts with proteins in this age group (Davydov et al., 2004, 2012). It can be explained by reducing rate of metabolic carbonyl products catabolism in enzymatic reactions in cytoplasm of myocytes during immobilization stress. Such alteration creates conditions for the stress damage of muscles under immobilization stress and contributes to the development of sarcopenia. A certain strain in the functioning of systems of protection of cells from free radical damage arises in myocytes myoplasm in pubertal animals also. However, it is pronounced to the lesser extent in comparison with old rats.

Comprehensive analysis of the results allow to conclude that adaptive changes preventing formation of oxidative stress and expression of its negative effects arise in skeletal muscle cells of adult animals in response to long-term immobilization. However, specificities in functioning of metabolic systems of anti-stress defense in cytosol and mitochondria develop in pubertal and old rats. Oxidative stress is manifested pronouncedly in mitochondria of pubertal animals during long-term immobilization. These stress manifestations serve as a background for alterations of pathways of utilization of cytotoxic free radical oxidation products. Despite the absence of such changes in cytosol of myocytes there are conditions for the stress damage of muscle tissue in pubertal rats.

Mitochondrial oxidative stress defense systems are suffi-

Table 2 Level products of free radical oxidation in postmitochondrial fraction of femoral muscle during stress (mean±SEM)

Age (months)	Experimental group of rats	Protein Carbonyls (μmol/mg protein)	TBA+ substances (μmol/mg protein)	Shiff Bases (nmol/mg protein)
1.5	Intact	0.61±0.14	0.045±0.006	0.48±0.04
	Immobilized	0.62±0.08	0.045±0.008	0.70±0.09
12	Intact	0.60±0.01	0.068±0.020	0.38±0.02
	Immobilized	0.64±0.01	0.028±0.004**	0.29±0.03**
24	Intact	0.57±0.13	0.050±0.016	0.23±0.02*
	Immobilized	0.73±0.13	0.025±0.005	0.20±0.03

* The data are positively distinguished from intact1.5-months-old rats ($p < 0.05$).
** The data are positively distinguished from intact rats of same age group ($p < 0.05$).
The table represents the average data from 6 investigations.

ciently effective in old rats under long-term immobilization conditions. However, direction of pathways of free radical oxidation carbonyl products catabolism alters in the cytoplasm of myocytes in old animals under immobilization stress. This fact contributes to the formation of metabolic preconditions for increase of sarcopenia manifestations during intense and long-term impact of the stressor.

Reasons of age-related changes of response of anti-stress systems in cytosol and mitochondria of skeletal muscles cells to long-term immobilization remain unclear. We can assume the relationship between their appearance and age-related peculiarities of endocrine regulation of antioxidant system and cell repair enzymatic systems under stress, as well as characteristic features of prooxidant effects in different compartments of the myocyte. Our future researches will be devoted to these aspects.

Compliance with ethics guidelines

Davydov V. V., Amjad Hamdallah, Grabovetskaya E. R. declare that they have no conflict of interest. All institutional and national guidelines for the care and use of laboratory animals were followed.

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