Induction of HSPs and Antioxidant Defense Enzymes during Activation of Free Radical Oxidation at the Early Stage of Hypokinesia

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We studied the stress component of the early stage of hypokinesia during hindlimb unloading. The intensity of free radical processes was evaluated and the content of protective proteins (antioxidant defense enzymes and proteins of the HSP family) was measured in the heart and liver. Three-hour hypokinesia increased the content of constitutive protective proteins, including hemoxygenase-2 and antioxidant defense enzymes, in the heart. Hypokinesia for 72 h was accompanied by more potent activation of antioxidant defense enzymes and increase in the content of inducible hemoxygenase-1, which leads to partial compensation of activated free radical oxidation. In the liver, hypokinesia of different duration suppressed the protective systems: the synthesis of inducible and constitutive hemoxygenases and antioxidant defense enzymes decreased, while the sensitivity of liver membrane structures to reactive oxygen species increased. We revealed a tissue-specific response to hypokinesia: pronounced damaging effect predominated in the liver and partial compensation of elevated production of reactive oxygen species was observed in the heart due to activation of protective systems.

Key Words: *free radical oxidation; antioxidant enzymes; HSP32; HSP70; hypokinesia*

Various pathological conditions, including stress, hypoxia, inflammation, hypothermia, fever, and physical exercise, are accompanied by not only specific response, but also a nonspecific reaction: accumulation of reactive oxygen species (ROS), which indirectly via redox signaling induce the synthesis of protective systems (antioxidant defense enzymes, protooncogenes, heat shock proteins, specific chaperons of membrane-bound enzymes, *etc*.).

Much attention was paid to the synthesis of inducible forms of various proteins of the HSP family, including HSP70 and HSP32 (hemoxygenase-1, HOx-1) in response to ROS signal under pathological conditions. The increase in superoxide radical content during stress and ischemic injury is a signal mechanism of the synthesis of HSP32 and HSP70 [6]. The level of HOx-1 depends on the concentration of oxygen and ROS in tissues. HOx-1 plays a regulatory role [6] and correlates with activity of antioxidant defense enzymes.

A tissue-specific balance between ROS and protective proteins exists in cells under physiological conditions. A shift in the balance toward activation of free radical processes under pathological conditions results in the synthesis of antioxidant defense enzymes and HSPs, which compensates for ROS excess. The degree of protective protein induction in a specific tissue is unknown. It remains unclear whether this induction can restore the physiological concentration of ROS. The knowledge of these mechanisms will allow us to realize additional exogenous protection of the most vulnerable organ under

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the influence of adverse factors (*e.g.*, stress). In our study the problem of tissue specificity was solved on the model of hypokinesia including the stress component at the initial stage. The urgency of this question is determined by high incidence of hypokinesia in medical practice (hindlimb fractures, cardiovascular, viral, and other diseases).

Hindlimb unloading causes muscular atrophy, increase in the volume of the connective tissue, transformation of slow muscle fibers to fast muscle fibers, and myofibrillar damage [1,7]. Recent studies showed that hypokinesia-induced atrophy of skeletal muscles and transformation of muscle fibers are accompanied by activation of free radical oxidation (*e.g.*, in the liver) [9,10]. However, little is known about these processes in the heart. It should be emphasized that free radical damage to the heart is induced by various stress factors. There are no data on the synthesis of immediate response protective proteins at the initial stage of hypokinesia characterized by significant stress reaction. The study of intracellular mechanisms of hypokinesia and the search for new methods of its correction are required to achieve improvement over the shortest period of rehabilitation after hypokinesia and avoid adverse effects on the heart and liver.

Here we evaluated the intensity of free radical processes and measured the content of protective proteins, antioxidant defense enzymes, and heat shock proteins in the heart and liver during hypokinesia of different duration.

MATERIALS AND METHODS

Experiments were performed on male Wistar rats weighing 250 g. Gravitational unloading was modeled by the standard method of antiorthostatic suspension (Morey—Holton). The rats were suspended in such a manner that the forelimbs contacted with the surface, while the hindlimbs remained unloaded. The rat tail was placed in special soft splints to prevent mechanical damage to the skin or compression of blood vessels. This experiment was conducted according to recommendations of the Committee on Biomedical Ethics (State Research Center of the Russian Federation, Institute of Medical and Biological Problems, Russian Academy of Sciences).

The animals were divided into groups (7 rats per group). Group 1 included control rats. Group 2 animals were subjected to hypokinesia (antiorthostatic suspension) for 3 h. Group 3 rats were subjected to 72-h hypokinesia. The animals were decapitated immediately after hypokinesia. The heart and liver were washed with physiological saline and frozen in liquid nitrogen. Heart tissue was homogenized in an Ultra-Turrax homogenizer (20,000 rpm, 3×30 sec) in a medium containing 20 mM Tris and 100 mM NaCl (pH 7.4) at 4°C (1:8 tissue/medium ratio). The homogenate was filtered through 2-layer gauze. The liver was homogenized in a Teflon-glass homogenizer (2×30 sec, 1:12 tissue/medium ratio).

The resistance of myocardial and liver tissue to free radical oxidation was estimated from accumulation of LPO products during *in vitro* induction in the system containing 0.5 mM ascorbate. Protein concentration did not exceed 3 mg/ml. The content of ROS-induced oxidation products was evaluated by measuring the maximum absorption of thiobarbituric acid-reactive substances (TBA-reactive substances) on a Hitachi-557 spectrophotometer as described elsewhere [8] with some modifications [3]. Activity of antioxidant defense enzymes was measured spectrophotometrically. Catalase activity was estimated by H_2O_2 consumption [5]. Superoxide dismutase (SOD) activity was determined from the difference between the rates of superoxide radical formation in the xanthin—xanthin oxidase system before and after addition of the sample [2]. Protein concentration was evaluated from the amplitude of the fourth derivative absorption spectrum at 240-320 nm.

The content of immediate response proteins HSP70, inducible and constitutive hemoxygenases (HOx-1 and HOx-2) in the cytosolic fraction was measured by Western blot analysis with monoclonal antibodies against HSP70 and HOx-1, polyclonal antibodies against HOx-2 (Stressgen), and peroxidase-labeled secondary antibodies (Jackson Immuno Research). Detection by chemiluminescence was performed with ECL reagents (Amersham) and X-ray film (Kodak).

Statistical treatment of data involved Statistica 6.0 software. Independent samples were compared by means of Mann—Whitney *U* test. The data are presented as the medians.

RESULTS

The expression of inducible HSP32 and HSP70 in the heart remained unchanged after 3-h hypokinesia (Fig. 1, *a*). At the same time, despite unchanged level of inducible HOx-1, the concentration of constitutive HOx-2 increased by 1.8 times. We hypothesize that the synthesis of low-molecular-weight and high-molecular-weight inducible proteins of the immediate response does not undergo significant changes over a short period (3 h), while constitutive hemoxygenase is always present in the

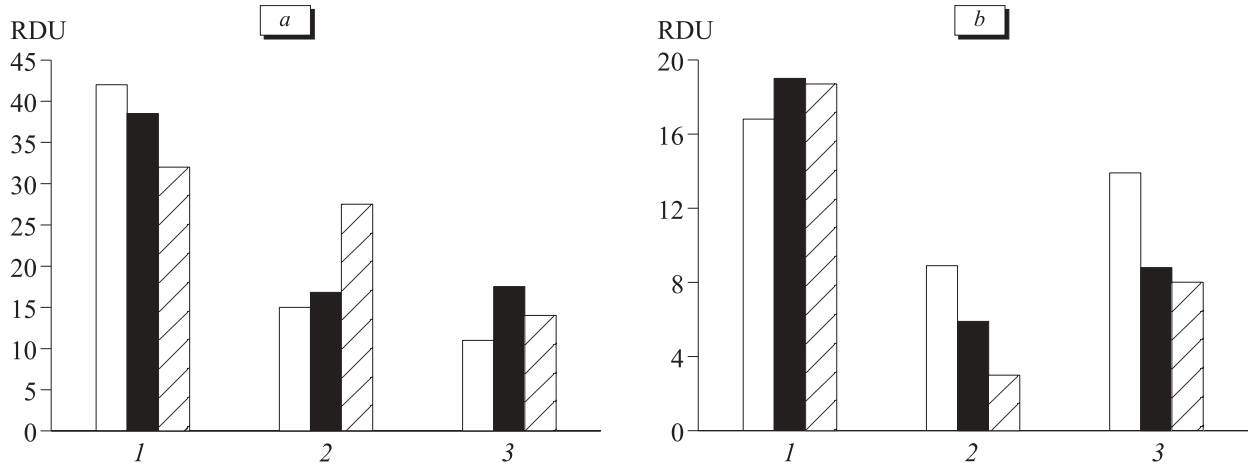

Fig. 1. Western blot analysis of inducible stress protein HSP70 (*1*), HOx-1 (*2*), and constitutive HOx-2 (*3*) in the heart (*a*) and liver (*b*) of control rats (light bars) and animals subjected to 3-h (dark bars) and 72-h hypokinesia (shaded bars). RDU, relative densitometric units.

organism and can react to the ROS signal. The increase of HOx-2 content probably reflects the rise in ROS level (*e.g.*, NO) after 3-h hypokinesia. It should be noted that HOx-2 serves as a NO-trapping agent.

The synthesis of inducible HOx-1 in the heart was noted after 72-h hypokinesia (Fig. 1, *a*). Since induction of HOx-1 synthesis is mediated by a change in the concentration of oxygen and ROS, the observed 2-fold increase in the expression of this protective protein suggests the appearance of a strong ROS signal and activation of the protective systems in cardiac cells after 72-h hypokinesia. HSP70 level in the heart did not increase compared to the control, which reflects partial compensation for the ROS signal and the absence of significant stress response by the 72nd hour of hypokinesia.

Therefore, the content of constitutive protective proteins of the HSP family increases at the early stage of hypokinesia. However, the synthesis of inducible forms is activated after 72 h. These changes suggest that ROS level increases after 3-h hypokinesia, and ROS signal remains high by the 72nd hour. The synthesis of protective systems continues in this period of the study.

For evaluation of this assumption we studied free radical oxidation and antioxidant defense system in the heart. Activity of antioxidant defense enzymes increased during hypokinesia. After 3- and 72-h hypokinesia catalase activity increased by 27 and 48%, respectively. SOD activity increased by 41% (Table 1). Catalase rapidly responds to acute treatments, which is consistent with the increase in enzyme activity during the early stage of hypokinesia. Catalase activity increased after 3-h hypokinesia and then almost 2-fold increased after 72-h hypokinesia in parallel with sharp activation of SOD. These changes are much more pronounced than the standard enzyme reaction to acute treatments.

The observed changes in antioxidant defense enzymes and protective proteins of the HSP family indicate that hypokinesia is accompanied by two processes in the heart: progressive increase in the synthesis of protective proteins over 72-h hypokinesia reflects the maintenance of the ROS signal. High activity of the antioxidant defense system is confirmed by strong activation of catalase, SOD, and HOx-1 after 72-h hypokinesia. The induction of free radical oxidation in the myocardium was studied to evaluate whether this protection can compensate for the ROS signal during 72-h hypokinesia.

The results of experiments with hypokinesia for 3 and 72 h are shown in Fig. 2, *a*. No intergroup differences were revealed in the basal level of oxidation products. The test for membrane resistance to free radical oxidation under *in vitro* conditions was performed to evaluate changes in heart tissue resistance to oxidation. After 3-h hypokinesia, the rate of oxidation product accumulation increased by 1.2-2.2 times and depended on the time of incubation. These changes reflect a sharp increase in heart tissue sensitivity to ROS. Increasing the time of hypokinesia to 72 h was not accompanied by further increase in tissue sensitivity and rate of induced free radical oxidation in the heart. The latter parameter did not differ after hypokinesia for 3 and 72 h. However, 72-h hypokinesia resulted in maximum activation of cell protection systems (antioxidant defense enzymes and heat shock proteins).

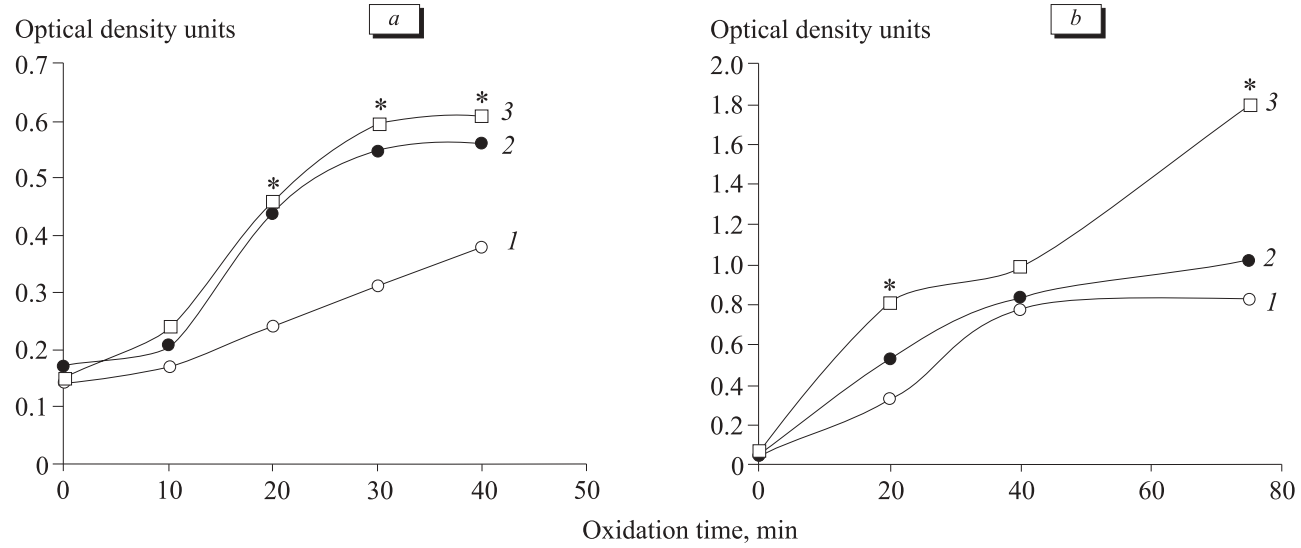

Fig. 2. Effect of hypokinesia of different duration on the accumulation of TBA-reactive substances during *in vitro* induction of free radical oxidation in the heart (*a*) and liver (*b*). Control (*1*), 3-h hypokinesia (*2*), and 72-h hypokinesia (*3*). *$p<0.05$ compared to the control.

Hence, short-term hypokinesia is followed by a significant increase in ROS level in muscle tissues. These changes are observed 3 h after the start of treatment. Heart sensitivity to free radical oxidation remains high after 3-day hypokinesia, which reflects the maintenance of the ROS signal. This exposure is characterized by the formation of the strongest protection from free radical oxidation. It is manifested in an increase in the content of protective proteins. Therefore, tissue sensitivity to ROS by the end of 72-h hypokinesia does not exceed that observed after 3-h treatment. High level of protective proteins partially compensates for hyperactivation of free radical processes in the heart after 72-h hypokinesia (adaptation to exposure).

Other results were obtained in studying the effect of hypokinesia on liver tissue. This organ is sensitive to the ROS signal. Function of the liver determines the response to various acute exposures, including stress and hypokinesia. Similarly to experiments with the heart, the content of protective proteins in the liver was measured after hypokinesia for 3 and 72 h. The liver reaction to hypokinesia was opposite and much more pronounced compared to that in the heart. The contents of constitutive and inducible hemoxygenase in the liver did not increase, but even decreased after 3-h exposure (Fig. 1, *b*). The inhibition of HOx-1 induction in the liver after 72-h hypokinesia was more pronounced compared to that observed by the end of 3-h exposure. Protein content decreased by more than 2 times (Fig. 1). These changes suggest significant activation of free radical oxidation, when ROS excess causes damage to various structures and components in the cell (*e.g.*, protective proteins). Our hypothesis is supported by the fact that the content of constitutive HOx-2 decreases by 34-44% after hypokinesia for 3 and 72 h. Similar results were obtained in measuring activity of antioxidant defense enzymes (Table 1). Catalase activity in the liver did not increase, while SOD activity significantly decreased.

Our results indicate that activation of the ROS signal in the heart during the early stage of hypo-

TABLE 1. Activity of Antioxidant Defense Enzymes after Adaptation to Hypokinesia

Group	Heart		Liver	
	SOD, arb. units/mg protein	catalase, μmol H_2O_2/min	SOD, arb. units/mg protein	catalase, μmol H_2O_2/min
1 (control)	4.81	2.17	7.53	27.57
2 (3-h hypokinesia)	4.7	2.75*	5.52*	29
3 (72-h hypokinesia)	6.8*	3.17*	5.35*	26.72

Note. *$p<0.05$ compared to the control.

kinesia is accompanied by the increase in activity of antioxidant defense enzymes and content of HSP proteins, which partially compensates for free radical oxidation after 72-h exposure. However, these processes do not occur in the liver. Activity of antioxidant defense enzymes and content of HOx-1 and HOx-2 in the liver decrease under these conditions. It can be suggested that free radical oxidation in the liver increases, while high signal of ROS is not compensated during hypokinesia.

Free radical oxidation was induced in the liver tissue to test this hypothesis. Figure 2, *b* illustrates the accumulation of free radical oxidation products in the liver during *in vitro* induction under control conditions and after hypokinesia for 3 and 72 h. The liver of animals subjected to 3-h hypokinesia was more sensitive to ROS compared to the control (by 1.7 times). The sensitivity of liver tissue to ROS increased to a greater extent after 72-h hypokinesia (2.6-fold higher compared to the control). It should be emphasized that the resistance of liver tissue after 72-h hypokinesia was much lower compared to that observed by the end of 3-h exposure. Therefore, ROS level remained high and was not compensated in this period.

We conclude that the significant increase in the content of protective proteins in the heart partially compensates for ROS-mediated processes. As distinct from the heart, the content of protective proteins in the liver decreases with increasing the duration of hypokinesia. These changes are accompanied by a progressive decrease in the resistance of liver membrane structures to ROS. Tissue-specific features of the cell response in various organs to hypokinesia, as well as the intracellular mechanisms of this reaction, should be taken into account to develop effective methods for the correction of this disorder.

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