

The Effects of Intermittent Hypoxia Training on Mitochondrial Oxygen Consumption in Rats Exposed to Skeletal Unloading

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Abstract. Intermittent hypoxia training (IHT) may reduce the oxidative stress-induced damage caused by extreme influences such as ischemia, exhaustive physical exercise, acute hypoxia, and stress. The aim of the present study is to investigate the effects of IHT on hepatic mitochondrial oxygen consumption, lipid peroxidation, and selected biochemical parameters used as diagnostic tools in a skeletal unloading model in rats. Our data showed that the IHT method significantly improved liver tolerance of unloading by reorganizing mitochondrial energy metabolism due to NADH-dependent oxidation. Aminotransferase activity was decreased compared to levels in untreated rats. Succinate dehydrogenase activity and lipid peroxidation remained unchanged when compared between groups. Moreover, skeletal unloading in the growing rats induced an activation of the rate of mitochondrial respiration in state 3 at succinate oxidation and decreased oxygen consumption at α -ketoglutarate oxidation. Adaptation of rats to IHT in our experiment significantly improved the rate of oxidative phosphorylation and the efficiency of phosphorylation in liver mitochondria at α -ketoglutarate oxidation. IHT seems to be a hepatoprotective method, and its use in maintaining a healthy liver and preventing unloading-induced liver damage deserves consideration and further examination.

Key words: intermittent hypoxia training, skeletal unloading, liver, mitochondria, oxidative phosphorylation, aminotransferases, succinate dehydrogenase, lipid peroxidation

Introduction

Mitochondrial dysfunction appears to be a possible mode of the molecular mechanism for oxidative stress [1,2]. Oxidative damage and the associated mitochondrial dysfunction may result in energy depletion, accumulation of cytotoxic mediators, and cell death [3]. The depletion of ATP levels and the changes in the activities of mitochondrial respiratory complexes indicative of lipid peroxidation and the reduction of oxidative phosphorylation efficiency have been implicated in stress-induced oxidative tissue damage [4,5]. Mitochondrial uncoupling has recently been identified as playing a determinant role in the pathogenesis of liver injury

due to the resulting defects in lipid homeostasis, bioenergetics impairment, and overproduction of reactive oxygen species (ROS) [2,6,7].

Most of the studies support a protective role of intermittent hypoxia training (IHT) [8-13]. Adaptation to hypoxia by an IHT is associated with the expression of enzyme isoforms that can efficiently function in a mitochondrial environment with high concentrations of reduced equivalents, as generated during hypoxia. This prevents inactivation of the mitochondrial enzyme complexes and may constitute one of the adaptation mechanisms triggered by IHT [8]. IHT enhances energy support production by increasing the rate of mitochondria formation, activating electron flux through mitochondrial respiratory complex I, and increasing the efficiency of oxidative phosphorylation [9-13]. Our previous study confirmed that

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IHT caused reorganization of mitochondrial energy metabolism and improved the protection against acute hypoxia favoring NAD-dependent oxidation [9-11]. After 14 days of normobaric IHT (11% O₂, 15-min sessions with 15-min rest intervals, 5 times daily), in comparison to controls, an acute hypoxic challenge in the presence of succinate resulted in an increase of the Chance respiratory coefficient, the ADP/O ratio and the phosphorylation rate, an increase in the activation of both aspartate and alanine aminotransferases, and in less lipid peroxidation. These findings indicate a more efficient use of oxygen under hypoxic conditions after IHT preconditioning [9,10]. In this study, we suggested an alternative approach to induce the self-protective system of an organism by applying a regime of adaptive training that combines periods of hypoxia and normoxia. IHT may reduce the oxidative stress-induced damage caused by extreme influences such as ischemia, exhaustive physical exercise, acute hypoxia, stress etc. [9]. We have assumed that the IHT method may induce the activation of various metabolic processes and may be an important trigger for specific adaptation, which improves the organism's resistance to simulated weightlessness modeled by skeletal unloading.

Weightlessness simulation is known to induce a number of physiological alterations [14]. Wronski and Morey-Holton's (1987) findings indicated that tail suspension may be a more appropriate model for evaluating the effects of simulated weightlessness on skeletal homeostasis [15]. Skeletal unloading in the growing rat induced a temporary inhibition of bone formation and thereby a deficit in bone calcium compared with age-matched, normally-loaded animals [16,17]. Dehority et al. (1999) demonstrated the utility of the adult rat hind limb elevation model as a means of simulating the loss of gravitational loading on the skeleton [18]. Chowdhury and Soulsby (2008) used a hind-limb suspended rat model of exposure to microgravity to monitor total tissue oxidative stress as a function of lipid peroxidation in liver, brain, heart, kidney, lung, pancreas, and small intestine homogenates in control loaded animals, in animals unloaded for two weeks, and in animals reloaded following two weeks of unloading. Results imply that

oxidative stress increases with the unloading of an animal, and it decreases toward control levels after two weeks of reloading following unloading [19].

The present study was thus designed to evaluate the effect of IHT on oxygen consumption data in the liver mitochondria of rats exposed to simulated weightlessness via the hind limb elevation model. It was further aimed to characterize the potential changes in the lipid peroxidation (thiobarbituric acid reactive substances content as important biomarkers of oxidative stress) and the alterations in selected biochemical parameters used as diagnostic tools in medicine (alanine and aspartate aminotransferases, and succinate dehydrogenase activities) in the liver tissue of rats exposed by a skeletal unloading model.

Materials and Methods

Animals. All procedures followed the criteria, technical standards, and rights applied to animal research as set forth in the statements of the European Union regarding the handling of experimental animals. This investigation conforms to legal and local ethical committee guidelines for animal research. Experiments were conducted on adult male Wistar rats (weight range 0.22-0.25 kg) provided with standard rat feed with water *ad libitum*. Animals were housed (4 per cage) in a room with 12:12-h light-dark cycle at 22°C.

Intermittent hypoxia training protocol. The IHT group included rats exposed to sessions of intermittent, normobaric hypoxia. We alternated repeated 15-min inhalations of a gaseous mixture containing 10% O₂ in N₂ with 15-min intervals of normoxia (21% O₂ in N₂) in a special Plexiglas chamber where the temperature and humidity were maintained at 22-23°C and 55-60%, respectively. Chamber O₂ content was monitored with O₂ analyzer. Animals had 5 similar sessions daily for 28 days.

Skeletal unloading procedure. The procedure of tail-suspension described by Wronski and Morey-Holton (1987) was adopted in the present study [15]. Animals were suspended using metal rings

with stainless steel implanted in the tail. Ring was implanted into tail of one-month rats. 1.5 months after implantation and the fibrous tissue formation around implanted ring, rats were subjected to the model with a head-down tilt for a two-week period. Total mechanical unloading of the hind limbs occurred. Animals were subjected in a 35° head-down tilt position, and the forelimbs maintained contact with the floor, allowing a 360° range of movement and access to food and water freely. The suspension height was adjusted to prevent the animal's hind limb from touching any supportive surface. The suspension height and animal behavior were monitored daily. Control animals were allowed to move unconstrained around the cages.

Experimental groups. The effect of IHT on oxygen consumption data in the liver mitochondria of rats exposed to simulated weightlessness through the hind limb elevation model was evaluated in three groups of animals: 1) the control group; 2) the group that experienced weightlessness simulation by mechanical skeletal unloading of the hind limbs during a two-week period; 3) the group that was treated with IHT daily for 28 days and then exposed to weightlessness simulation for a two-week period. Each experimental group consisted of 6 rats. Animals were lightly anesthetized with ether and then decapitated immediately after the experiment.

Isolation of liver mitochondria. The liver was removed immediately from each rat after decapitation. One liver was used for each mitochondrial preparation. Mitochondria were isolated by differential centrifugation according to the method described by Kondrashova and Doliba (1989) [20]. Briefly, the liver was excised, weighed, and washed in ice-cold buffer. The minced tissue was rinsed clear of blood with cold isolation buffer and homogenized in a glass Potter-Elvehjem homogenizing vessel with a motor-driven Teflon pestle on ice. The isolation buffer contained 120 mM KCl, 2 mM K_2CO_3 , 10 mM HEPES, and 1 mM EGTA. Potassium hydroxide was used to adjust the pH of the medium to 7.20. The suspension was then centrifuged for 5 min at 600 g at 0°C. The mitochondrial fraction was obtained by centrifugation of

supernatant for 12 min at 8,000 g at 0°C. Finally, mitochondria were re-suspended in the isolation buffer at a concentration of 4 to 6 mg of protein per mL, and were kept in the tube on ice until polarographic measurements were performed. The protein concentration in each sample was determined according to Bradford's method (1976) using bovine serum albumin as a standard [21]. There were 6 mitochondrial preparations in each experimental group.

Measurement of oxygen consumption.

Mitochondrial respiratory function was measured in the multichannel chamber, using a Clark-type electrode, by the polarographic method of Chance and Williams (1956) [22]. Mitochondria were added to the respiration chamber containing a total volume of 1.0 mL of incubation media. The medium contained 120 mM KCl, 2 mM K_2CO_3 , 2 mM KH_2PO_4 , 10 mM HEPES. Potassium hydroxide (1.0 N) was used to adjust the pH of the medium to 7.20 at 26°C. Succinate (0.35 mM final concentration) and α -ketoglutarate (1 mM final concentration) were used as oxidative substrates. Glutamate (3 mM final concentration) and malate (2.5 mM final concentration), as well as glutamate (3 mM final concentration) and pyruvate (3 mM final concentration) mixtures were used as donors of α -ketoglutarate through aminotransferases reaction. An inhibitor of succinate dehydrogenase, malonic acid (2 mM final concentration), was used to assess the presence of FADH-dependent substrates in the mitochondrial oxidation. ADP (phosphate acceptor) was administered at a concentration of 0.2 mM.

Mitochondrial oxygen consumption parameters measured were the following: State 2 (oxygen consumption before the addition of ADP), State 3 (oxygen consumption stimulated by ADP), State 4 (oxygen consumption after cessation of ADP phosphorylation), respiratory control ratio (RCR) described by Chance (ratio of state 3 to state 4), the ADP/O ratio (ratio between the nanomoles of ADP phosphorylated and the nanomoles of oxygen consumed during state 3), and the rate of phosphorylation (Vph). The respiratory control ratio and ADP/O ratio were calculated by the method of

Chance and Williams (1956). Oxygen consumption was determined in the presence (state 3) or in the absence (state 4) of phosphate acceptor and recorded as nanogram oxygen atoms per minute per milligram of mitochondrial protein. The respiratory control ratio by Chance was calculated as the ratio of state 3 to state 4 respiration rates. The ADP-to-oxygen-ratio (ADP/O) was calculated as the ratio of nanomoles of added ADP per nanogram atoms of oxygen utilized during state 3 [22].

Biochemical assay. TBARS assay for lipid peroxidation. The malondialdehyde (MDA) content, a measure of lipid peroxidation, was assayed in the form of thiobarbituric acid reacting substances (TBARS) by the method of Kamyschnikov (2004) [23]. Briefly, to 1.0 mL of 20% trichloroacetic acid (TCA) reagent were added 2.1 mL of liver homogenate 10% (w/v), and 0.8% aqueous solution of 2-thiobarbituric acid. The mixture was heated at 100°C for 10 min. After cooling with ice-cold water, the mixture was centrifuged at 3,000 g for 10 min. The supernatant was removed and its absorbance was measured at 540 nm. TBARS were quantified using an extinction coefficient of $1.56 \cdot 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ and expressed as μmol of MDA per mg protein.

Alanine aminotransferase (ALT, E.C. 2.6.1.2), aspartate aminotransferase (AST, E.C. 2.6.1.1) activities assay. ALT and AST, the ketoacids produced by the enzyme action, react with 2,4-dinitrophenylhydrazine, producing a hydrazone complex measured calorimetrically at 530 nm by the method of Reitman and Frankel (1957) [24]. ALT and AST activities were expressed as μmol pyruvate per h per mg of protein.

Succinate dehydrogenase (SDH, E.C. 1.3.5.1) activity assay. SDH activity was analyzed spectrophotometrically by the method of Eschenko and Volski (1982) [25]. The enzyme assay mixture consisted of 1 mL 0.1M phosphate buffer (pH 7.8), 0.1 mL of 0.1M succinic acid (pH 7.8), 25 mM EDTA (pH 7.8), and 150 mM sodium aside. 0.5 mL of 10% liver homogenate was then added to the mixture. The reaction was initialized by adding 0.1 mL of 25 mM $\text{K}_3[\text{Fe}(\text{CN})_6]$ and incubating for 15

min at 30°C. The reaction was terminated by the rapid addition of 2.0 mL of 20% TCA reagent. TCA was added to blank assay before tissue homogenate. All samples were centrifuged at 3,000 g for 10 min. The absorbance of the obtained supernatant was measured at 420 nm and was compared with that of the blank. One unit of SDH activity is defined as the amount of enzyme required for decomposition of 1 nmol succinic acid per min per mg of protein.

Statistical analysis. Results are expressed as mean $\pm$ S.E.M. All variables were tested for normal distribution using the Kolmogorov-Smirnov test ($P > 0.05$). The significance of differences in the TBARS level, enzyme activities, and oxygen consumption data (significance level at $p < 0.05$) was examined using the Mann-Whitney U test according to Zar (1999) [26]. In addition, the relationships between TBARS level, enzyme activities, and oxygen consumption data of all individuals were evaluated using Spearman's correlation analysis. All statistical calculations were performed on separate data from each individual with STATISTICA 8.0 software (StatSoft, Poland).

Results

Table 1 shows the mitochondrial oxygen consumption data in rat liver using FADH- (succinic acid) and NADH-dependent substrates (α -ketoglutarate) in the skeletal unloading model and under IHT influence. In order to determine the effect of IHT on mitochondrial oxygen consumption data, the skeletal unloading model was tested.

Skeletal unloading induced a considerable increase of 31.9% ($p = 0.045$) in the V_3 value at succinate oxidation compared to the control group. More significant changes in the mitochondrial oxygen consumption data have been detected for α -ketoglutarate oxidation. Skeletal unloading decreased the RCR by 45.1% ($p = 0.037$) and the efficiency of phosphorylation by 113% ($p = 0.037$) (**Table 1**). In IHT treated rats exposed to the skeletal unloading model, the oxidation of NADH-dependent substrate α -ketoglutarate suffered no changes in the rate of respiration in state 3, except

Table 1. Effect of the skeletal unloading model on liver mitochondrial oxygen consumption data in IHT treated rats under various substrates oxidation (mean±S.E.M., n=6).

Group of animals	V ₃ , ng O•min ⁻¹ •mg ⁻¹ protein	RCR, V ₃ /V ₄	ADP/O, μmol ADP•ng ⁻¹ O	Vph, μmol ADP•min ⁻¹ • mg ⁻¹ protein
0.35 mM succinate				
Control	54.27±4.87	3.40±0.44	1.76±0.15	94.62±9.69
Skeletal unloading model	71.58±6.48*	4.20±0.51	2.07±0.14	148.88±17.81
IHT and skeletal unloading	61.53±6.52	4.20±0.43	1.83±0.05	112.87±13.14
1 mM α-ketoglutarate				
Control	36.47±3.43	4.79±0.83	2.30±0.18	83.34±9.81
Skeletal unloading model	42.42±6.24	2.63±0.50*	1.81±0.05*	77.0±11.66
IHT and skeletal unloading	55.52±4.10	2.59±0.46	2.39±0.20**	133.74±17.98*
1 mM α-ketoglutarate+2.5 mM malonate				
Control	31.64±2.89	2.96±0.42	1.60±0.19	50.68±7.32
Skeletal unloading model	41.98±4.77	3.37±0.43	1.76±0.10	73.39±8.26
IHT and skeletal unloading	36.93±3.38	2.45±0.41	2.10±0.11	77.97±9.05
3 mM glutamate+2.5 mM malat				
Control	68.25±4.35	3.23±0.19	1.69±0.10	116.63±12.18
Skeletal unloading model	80.31±5.01	3.35±0.33	1.79±0.08	144.03±11.36
IHT and skeletal unloading	108.94±6.91**	4.30±0.44	1.92±0.21	211.98±32.44
3 mM glutamate+3 mM pyruvate				
Control	59.55±6.37	3.64±0.52	1.81±0.22	114.54±24.74
Skeletal unloading model	93.78±8.23*	3.33±0.39	2.29±0.08	214.76±19.98*
IHT and skeletal unloading	108.61±9.87	3.82±0.38	1.94±0.16	208.83±24.05

* p<0.05, Skeletal unloading group was compared to the untreated group (as Control).

** p<0.05, IHT and skeletal unloading was compared to the skeletal unloading group.

for significant increases in ADP/O (by 32%, p=0.010) and Vph (by 73.7%, p=0.001) compared to the skeletal unloading group. IHT treatment also significantly increased the rate of mitochondrial respiration in state 3 (by 35.6%, p=0.016) for α-ketoglutarate oxidation via transaminase reaction (glutamate and malate oxidation). Skeletal unloading of IHT-treated rats modified the mitochondrial oxygen consumption data by increasing mitochondrial respiration in state 3 by 57.5% (p=0.006) and increasing the rate of phosphorylation by 87.5% (p=0.025) at α-ketoglutarate oxidation through transaminase reaction (glutamate and pyruvate oxidation) (**Table 1**).

Statistically significant differences in ALT and AST activity were observed between rats from control

and skeletal unloading groups. ALT and AST activity was decreased by 42.9% (p=0.004) and by 51.3% (p=0.004), respectively, in the skeletal unloading groups compared to the control group. SDH activity and lipid peroxidation remained unchanged when compared between groups (**figures 1, 2 and 3**).

Several correlations between checked parameters were found (**Table 2**). Liver SDH activity from the untreated group was inversely correlated with the ADP/O ratio when α-ketoglutarate was used as a substrate (R=-0.829, p=0.042). AST activity from the skeletal unloading group correlated positively with V₃ (R=0.829, p=0.042) and RCR (R=0.886, p=0.019) at α-ketoglutarate oxidation. The relationship between AST activity and the

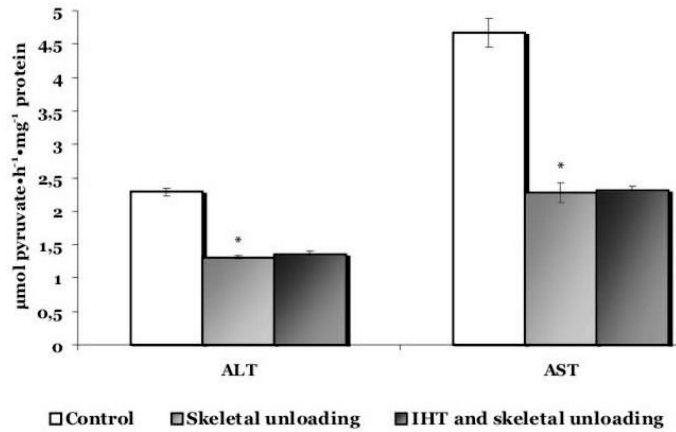


Figure 1. Effect of IHT treatment on alanine- and aspartate aminotransferases activity ($\mu\text{mol pyruvate} \cdot \text{h}^{-1} \cdot \text{mg}^{-1} \text{ protein}$) in the liver tissue of rats exposed to skeletal unloading model.

Each bar represents the mean $\pm$ S.E.M.

* $p < 0.05$, Skeletal unloading group was compared to the untreated group (as Control).

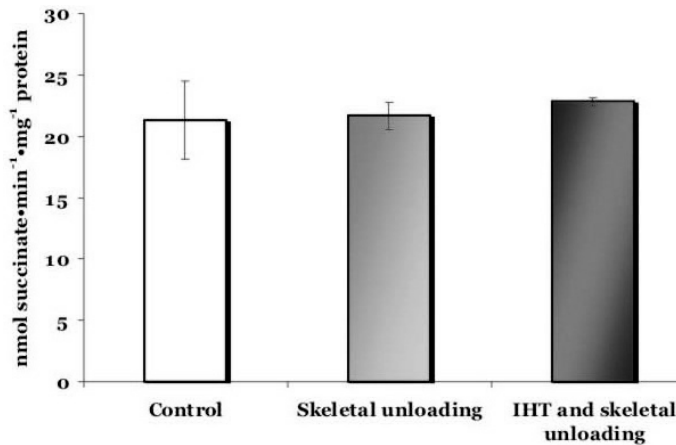


Figure 2. Effect of IHT treatment on succinate dehydrogenase activity ($\text{nmol succinate} \cdot \text{min}^{-1} \cdot \text{mg}^{-1} \text{ protein}$) in the liver tissue of rats exposed to skeletal unloading model.

Each bar represents the mean $\pm$ S.E.M.

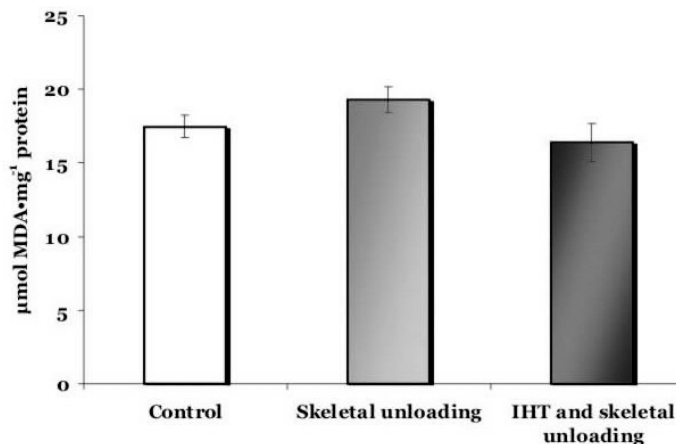


Figure 3. Effect of IHT treatment on TBARS level ($\mu\text{mol MDA} \cdot \text{mg}^{-1} \text{ protein}$) in the liver tissue of rats exposed to skeletal unloading model.

Each bar represents the mean $\pm$ S.E.M.

ADP/O ratio at glutamate and pyruvate transamination pathway ($R=0.943$, $p=0.005$) was positive in the liver of rats treated with IHT and exposed to an unloading model.

Discussion

This study confirmed that IHT improves liver tolerance to simulated weightlessness modeled by skeletal unloading. We have demonstrated for the first time that the exposure of rats to IHT induced protection against skeletal unloading. We examined one of the physiologic mechanisms responsible for such improvement and provided conclusive evidence that IHT attenuated the derangements in mitochondrial respiration associated with unloading. Skeletal unloading caused impairment of the RCR and ADP/O ratio that used α -ketoglutarate as a substrate, and increased oxygen consumption (state 3) at succinate oxidation. In our study, oxidation of α -ketoglutarate, an NADH-dependent substrate, was inhibited. RCR, which provides an index of mitochondrial coupling [22, 27, 28], was decreased after the unloading exposure. The skeletal unloading caused the decline of mitochondrial consumption when NADH-dependent substrate (α -ketoglutarate) was used. Those changes were completely absent when an NADH-dependent substrate was used due to glutamate and pyruvate transamination pathway. Our findings are in accordance with previous reports, demonstrating that stress-induced adrenaline prevalence leads to the increase of succinate-dependent ATP synthesis, which is due to the well-known activation of succinate oxidation [29]. The results of Fedotcheva et al. (2006) suggest that nonenzymatic decarboxylation of α -ketoglutarate and transamination switch the tricarboxylic acid cycle to the formation and oxidation of succinate, bypassing NADH-dependent oxidation, and thereby supporting

Table 2. Correlative analysis of mitochondrial respiration data, enzymes activity and lipid peroxidation in the liver of untreated rats, rats exposed to skeletal unloading, and IHT treated rats.

Relation	Correlative coefficient, R	Significant difference level, p Control group
ADP/O (α -ketoglutarate oxidation)–SDH	-0.829	0.042
V3/V4 (α -ketoglutarate+malonate)–SDH	-0.943	0.005
V3/V4 (α -ketoglutarate oxidation)–TBARS	-0.829	0.042
Skeletal unloading group		
ADP/O (glutamate+malate)–ALT	-0.829	0.042
V3 (α -ketoglutarate oxidation)–AST	0.829	0.042
V3/V4 (α -ketoglutarate oxidation)–AST	0.886	0.019
ADP/O (glutamate+malate)–SDH	0.943	0.005
SDH–AST	-0.829	0.042
IHT and Skeletal unloading group		
ADP/O (glutamate+pyruvate)–AST	0.943	0.005
ADP/O (succinate oxidation)–SDH	-0.886	0.019
ALT–SDH	-0.886	0.019

respiration [30]. Transamination supplies α -ketoglutarate and reduces the sensitivity of NADH-dependent oxidation to reactive oxygen species. The transamination pathway can operate under oxidative stress and is remarkably resistant to oxidants [30].

In our study IHT caused reorganization of mitochondrial energy metabolism, favoring NADH-dependent oxidation and improving protection against skeletal unloading. IHT treatment could slightly improve the energy support processes in liver mitochondria, by increasing efficiency and by increasing the phosphorylation rate at oxidation of NADH-generated substrates. Both the rate of ADP-stimulated oxygen consumption (state 3) and the rate of phosphorylation (Vph) in the livers of the IHT and unloading groups were decreased by 33.5% ($p=0.010$) and by 41.7% ($p=0.016$) respectively, using α -ketoglutarate as a substrate and malonate as an SDH inhibitor, compared to α -ketoglutarate oxidation. Because a malonate-sensitive component in α -ketoglutarate oxidation induces the decrease of phosphorylation efficiency compared to α -ketoglutarate oxidation, these data presume the regulatory role of succinate in

oxidation of NADH-generated substrates. On the other hand, IHT elicited an activation effect on respiratory capacity and phosphorylation rate at oxidation of glutamate and pyruvate (formation of α -ketoglutarate through transamination).

The influence of sympathetic and parasympathetic systems on secretory cell respiration has been shown to be mediated by adreno- and cholinoreceptors of plasma membranes and is related to selective oxidation of two different substrates of the Krebs cycle: succinate and α -ketoglutarate [31]. Shostakovskaia et al. (1986) demonstrated that intraperitoneal acetylcholine injection induced activation of α -ketoglutarate oxidation in the rat liver mitochondria [32]. This mediator activates M-cholinoreceptors causing distinct stimulation of phosphorylation respiration rate and increasing the calcium capacity of mitochondria with α -ketoglutarate oxidation. Atropine, an acetylcholine inhibitor, completely removed this effect [32]. Administration of noradrenaline induced activation of SDH in the rat liver and is mediated by cAMP [33]. Doliba et al. (1993) [34] and Hordii et al. (1994) [31] hypothesized that the enzyme–substrate–nucleotide interaction plays a critical role in the mechanisms of stress

adaptation [31,34]. According to this hypothesis, mitochondrial α -ketoglutarate and succinate oxidation is included in the composition of two reciprocal hormonal–substrate–nucleotide systems: acetylcholine– α -ketoglutarate–cGMP–GTP and catecholamines–succinate–cAMP–ATP.

Reciprocity of these systems is necessary for regulation of cell oxygen demand and effective oxygen utilization for ATP-synthesis and synthesis of other macroergical compounds, depending on the functional state of a cell [31]. Moreover, nonenzymatic oxidation of α -ketoglutarate can support an abbreviated tricarboxylic acid cycle, provided that the supply of α -ketoglutarate is renewed through transamination [35]. It is possible that IHT causes α -ketoglutarate oxidation and improves protection against skeletal unloading due to activation of parasympathetic systems by activation of cholinergic receptors.

Our findings are in accordance with the previous reports demonstrating an activation of respiration by IHT [36]. For example, Kurhaliuk et al. (2002) showed that IHT caused oxidation of NADH-dependent substrates and an improvement of protection against acute hypoxia and inhibition of lipid peroxidation in liver mitochondria [10]. Furthermore, they found that intermittent hypoxia improved energy producing metabolic processes by activating electron flux through mitochondrial respiratory complex I, and increasing the efficiency of oxidative phosphorylation [8–10]. These effects are mediated partly by NO-dependent reactions [37,38]. The results of Fedotcheva et al. (2006) also suggest a mechanism for succinate-dependent regulation of hypoxia-inducible factors (HIF) via the nonenzymatic oxidation of α -ketoglutarate to succinate under oxidative stress. ROS, induced under normoxic and hypoxic conditions, could disrupt tricarboxylic acid cycle reactions and promote the nonenzymatic conversion of α -ketoglutarate to succinate [30,35]. Both metabolites, α -ketoglutarate and succinate, participate in the regulation of transcription activity of HIF-1 α [30].

Previous studies have shown that IHT also exerts direct effects, decreasing the pronouncement of tissue hypoxia and intracellular acidosis under acute hypoxic conditions, improving O₂ transport in tissues, increasing oxidative metabolism, enzyme synthesis and ion-transport systems in cell membranes, and rearranging the composition of phospholipids in cell membranes [38,39]. Adaptation to stress results in enhanced expression of stress proteins and antioxidant systems that can then provide protection against generalized stress and disease [40,41]. Meerson et al. (1996) suggest that positive IHT effects are based on the cross-protection values of adaptations to one stress providing resistance to another stress. It was shown that the influence of dosed periodic hypoxic training can be beneficial to the processes of mitochondrial energy support under stressed conditions and limit the enhancement of stress hormone concentrations on the central and peripheral level [42]. Our previous studies demonstrated that IHT, through the release of nitric oxide, could modulate the activity of the mitochondrial respiratory chain by activating electron flux through respiratory complex I and increasing oxidative phosphorylation efficiency [9,10].

Lukyanova et al. (2007) demonstrated that the basic molecular response to intermittent normobaric hypoxia on the formation of adaptive signs in the cerebral cortex of rats with different resistance to hypoxia involves the mitochondrial enzyme complexes [43]. There is evidence that less resistant animals were characterized by more effective functioning of rotenone-sensitive NADH-cytochrome c reductase and succinate-cytochrome c reductase under conditions of increased reduction status of the cell. Reciprocal relations between mitochondrial enzyme complexes in the substrate region of the respiratory chain play a role in the signal regulatory mechanism, which mediates tissue-specific and general resistance of rats under conditions of intermittent normobaric hypoxia [44]. Lukyanova (2011) recently described major mechanisms of urgent adaptation to hypoxia by succinate as a signaling molecule whose effects are carried out at

three levels [45]. First, transient, reversible, compensatory activation of respiratory chain complex II is necessary for succinate-related energy synthesis in conditions of oxygen deficiency and formation of urgent resistance in the body (intercellular effect). Second, succinate stabilized HIF-1 and initiates its transcriptional activity related with formation of long-term adaptation (intracellular effect). Third, succinate activates a succinate-specific receptor CPR91 (intramitochondrial effect). In the cascade of hypoxia-induced metabolic alterations, rotenone-sensitive NADH-dependent oxidation pathway is most sensitive to intracellular oxygen deficiency [45].

A number of studies indicate that hypoxia induces reprogramming of the respiratory chain function and switching from oxidation of NADH-dependent substrates (complex I) to succinate oxidation (complex II) [45]. Inhibition of complex I of the mitochondrial electron transport chain appears to be one of the sources for IHT-induced generation of ROS [36]. The reversible inhibition of mitochondrial complex I leads to both suppression of reduced equivalent flux through the NADH-dependent site of the respiratory chain and the emergency activation of compensatory metabolic pathways, primarily the succinate oxidase pathway [8]. Intensification of hypoxia leads to propagation of the pathological process toward the cytochromes in the respiratory chain. Electron transport in the area of the b-c cytochromes becomes inactivated first. Only in the vicinity of anoxia is cytochrome c oxidase inhibited [8].

In summary, this study demonstrates that IHT has potent protective effects against hepatic damage in rats exposed to skeletal unloading. Here we demonstrated that the IHT method improved liver tolerance to unloading by reorganization of mitochondrial energy metabolism due to NADH-dependent oxidation. IHT caused an elevation in mitochondrial respiration (ADP/O ratio and rate of phosphorylation) at α -ketoglutarate oxidation. IHT seems to be a potent hepatoprotective method and its use in the maintenance of a healthy liver and in the prevention of unloading-induced liver damage deserves consideration and further examination.

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