

Effect of intermittent hypoxia on pro- and antioxidant balance in rat heart during high-intensity chronic exercise

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The purpose of the present study was to elucidate the influence of sessions of intermittent hypoxic training (IHT) alone and in combination with high-intensity chronic exercise on lipid peroxidation and antioxidative defense system in rat heart. High-intensity chronic exercise was performed as swimming training with load that corresponded to $\sim 75\%$ VO_{2max} (30 min/day, 5 days/wk, for 4 wk). IHT consisted of repeated episodes of hypoxia (12% O_2 , 15 min), interrupted by equal periods of recovery (5 sessions/day, for 2wk). Sessions of IHT were applied during the first two weeks and during the last two weeks of chronic exercise. It was shown that long-term training was accompanied by the accumulation of thiobarbituric acid reactive substances (TBARS) in myocardium. IHT attenuated the increase in TBARS content caused by high-intensity chronic exercise and it enhanced myocardial reduced glutathione concentration, activities of superoxide dismutase, catalase, and glutathione peroxidase in comparison with trained animals only. No significant changes were found in glutathione reductase, glucose-6-phosphate dehydrogenase activities. Our results suggest that intermittent hypoxic stimuli may induce a state of preconditioning that protects the heart from oxidative stress evoked by high-intensity chronic exercise.

Keywords: intermittent hypoxic training, chronic exercise, oxidative stress, antioxidative system, heart

In the latest years evidence has accumulated on the positive influence of intermittent hypoxia (IH) on respiratory system, organism oxygen regimens, metabolic and structural reconstruction in different human and animal tissues (4, 9, 24). In cardiac muscle, intermittent hypoxic exposure may reduce the risk of coronary heart disease, prevent the onset of arrhythmias (3), and improve myocardial tolerance to chronic hypoxia-induced dysfunction (24). Experimentally repeated short-term hypoxia with

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normoxic intervals is also known as intermittent or interval hypoxic training (IHT) (4, 5). IHT increases the hypoxic ventilatory response, red blood cell mass, erythropoietin level, and aerobic capacity (5). Some of these effects might be potentially beneficial in sports training, for acclimatization to high altitude, for treatment of various illnesses, and for correction of pathologic conditions (16, 18, 37). Effects of IHT are much more pronounced in situations promoting oxidative stress (4, 16). It was reported that exercise under hypoxic conditions could induce muscular and systemic adaptation (9), increase of vascular endothelial growth factor (VEGF) mRNA, capillarity and myoglobin mRNA, oxidative enzymes activity in tissues that improved aerobic performance and exercise tolerance (16, 36, 37).

It is known that heart is an aerobic organ with a high oxidative metabolism level and as a consequence, with a great oxygen consumption rate, which significantly increases during physical exercise (14). Many studies have reported that strenuous or endurance exercise may cause oxidative stress and heart tissue damage (22, 34). As a defensive strategy, myocytes are capable of inducing antioxidant enzymes and antioxidants such as superoxide dismutase (SOD), catalase (CAT), reduced glutathione (GSH) and GSH-related enzymes – glutathione peroxidase (GPx), glutathione reductase (GR), glucose-6-phosphate dehydrogenase (G6PDH), to remove the excess of reactive oxygen species (ROS) (15, 33). At the same time, myocardial antioxidant enzymes activity and GSH content are rather modest, comparable to those in liver and soleus muscle (19, 34) and the heart is vulnerable to chronic exercise-induced oxidative stress (14, 15). Recent studies have demonstrated the influence of IHT on intracellular prooxidant-antioxidant homeostasis (4, 12), however, evidence concerning the effects of IHT on antioxidant systems of heart muscle especially under physical exercise is insufficient.

The purpose of the present study was to determine the influence sessions of IHT at rest and in combination with long-term high-intensity swimming exercise on the balance between prooxidants and antioxidants in rat heart tissue.

Materials and Methods

The chemicals applied for biochemical assays were purchased from Sigma, Fluka, and Merck. The protocol of this study was approved by the local Animal Research Ethic Committee. Male Wistar rats (3 months of age at the beginning of the experiment) were used. Rats were fed with standard laboratory chow and water ad libitum and kept under artificial light-dark cycle of 12 h. The rats were randomly divided into groups as follows:

Group 1, normal control (n=10)

These rats were sedentary and under normoxic condition.

Group 2, acute exercise (n=10)

In this group, animals were subjected to a single 30-minute acute swimming exercise with load that was $10 \pm 1.2\%$ of the body weight. The load was selected individually for each rat and attached to the root of the tail.

Group 3, chronic exercise (CE) (n=10)

These rats were subjected to the endurance training program consisting of swimming with load that was $10 \pm 1.2\%$ of the body weight for 30 min/day, 5 days/wk, during 4 wk. The load was selected individually for each rat every day. The training intensity at this level corresponded to $\sim 75\%$ maximal oxygen consumption (VO_{2max}) and was maintained for 4 weeks.

Group 4, intermittent hypoxic training (IHT) (n=10)

These animals were subjected to intermittent hypoxic training for two weeks. Hypoxic episodes were created by breathing hypoxic gas mixtures (12% O_2) under normobaric condition in a special chamber. During the experiment we applied repeated short-term hypoxia (15 minutes) with normoxic intervals (15 minutes). Rats had five such sessions daily.

Group 5, CE + IHT during the first two weeks (n=10)

In this experimental group, animals were subjected to a high-intensity chronic exercise for 4 weeks in association with sessions of interval hypoxic training during the first two weeks of the swimming program.

Group 6, CE + IHT during the last two weeks (n=10)

In this experimental group, animals were subjected to a high-intensity chronic exercise for 4 weeks in combination with sessions of interval hypoxic training during the last two weeks of swimming program. Rats in groups 5 and 6 had a swimming training program and sessions of interval hypoxic training similar to groups 3 and 4. Swimming exercises were performed in a beaker (50 cm in depth and 50 cm in width) that was submerged in a thermostatic water bath set at $37^\circ C$. Rats swam in groups of 2–3 because this stimulated the animals to the exercise more rigorously.

VO_{2max} was measured according to Brooks and White (6). VO_{2max} was defined as the VO_2 after which an increase in work rate was not associated with a further increase ($\pm 5\%$) in VO_2 . At the beginning of the swimming regimen, the mean rat weights of the various groups did not differ significantly.

After the acute exercise, the animals were killed immediately by decapitation. In the other experimental groups, animals were killed 24 h after the last exercise training session. At the time of sacrifice, the animals were lightly anaesthetized with ether. The heart was quickly removed and placed in liquid N_2 . Samples of the tissue were washed in cold saline and homogenized in ice-cold 10 mM phosphate buffer (pH 7.4) (1:10 w/v). The homogenates were centrifuged at 15 000 g for 30 min and the supernatant was used for enzymes activity and lipid peroxidation assays. All procedures

were carried out at 0–4 °C. Lipid peroxidation was carried out by the measurement of the thiobarbituric acid reactive substances (TBARS) following the method described by Ohkawa et al. (27). Total superoxide dismutase (SOD; EC 1.15.1.1) activity was determined by the method of Misra and Fridovich (25). Catalase (CAT; EC 1.11.1.6) was assayed by the technique described by Aebi (1). Reduced glutathione (GSH) content was estimated by the method of Sedlak and Lindsay (32) and based on the development of the stable yellow color with Ellman's reagent (5',5'-dithiobis-(2-nitrobenzoic acid)). Glutathione reductase (GR; EC 1.6.4.2) was measured by the NADPH extinction decrease at 340 nm by the method of Carlberg and Mannervik (8). Glutathione peroxidase (GPx; EC 1.11.1.9) activity was determined as described by Olinescu and Nita (28). Glucose-6-phosphate dehydrogenase (G6PDH; EC 1.1.1.49) activity was determined by Deutsch (11). The protein content was determined by the method of Lowry et al. (23) using bovine serum albumin as a standard.

Results are given as means \pm SEM (standard error of the means) and the data were analyzed using Student's *t*-test for significant differences between experimental groups and their corresponding control groups. A level of $p < 0.05$ was accepted as statistically significant.

Results

Acute high-intensity exercise induced a 13% increase ($p < 0.01$) of the TBARS content in heart in comparison with sedentary rats (Fig. 1A). Simultaneously we registered reduction in activities of SOD, GR, GPx and decrease in GSH content. Single acute swimming exercise caused an increase in CAT and G6PDH levels, but these changes were not statistically significant (Fig. 1B, C). Chronic high-intensity exercise induced a decrease in TBARS content in myocardium in comparison with group 2 ($p < 0.01$), at the same time the concentration of TBARS remained higher than the control level ($p < 0.01$). No significant changes were found in SOD and CAT activities. Myocardial GSH content, GR, G6PDH levels were increased in comparison with sedentary rats ($p < 0.05$). Trained rats demonstrated only a tendency towards an increase in GPx activity (Table I). After sessions of IHT the concentration of GSH, activities of SOD, CAT, GPx were increased as compared with normoxic rats ($p < 0.05$) (Table I; Fig. 1B, C). We registered these changes in antioxidative enzymes activity in cases when TBARS content was slightly increased ($p < 0.05$). Sessions of IHT in combination with chronic exercise demonstrated decrease in TBARS content in comparison with trained rats only ($p < 0.05$) (Fig. 1A). In rats under different regimens of IHT myocardial SOD, CAT, and GPx activities as well as GSH content were markedly higher than in normoxic and chronically trained rats ($p < 0.05$) (Fig. 1B, C). IHT in combination with swimming slightly promoted the increase of G6PDH activity in comparison with control animals. However, these changes were not statistically significant (Table I).

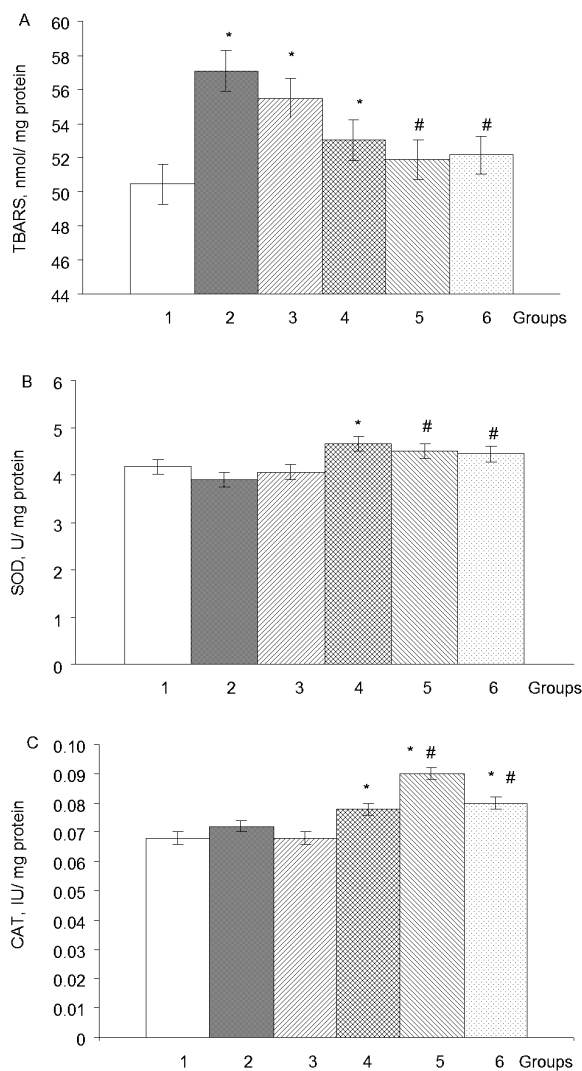


Fig. 1. Changes in thiobarbituric acid reactive substances (TBARS) content (A), activity in superoxide dismutase (SOD) (B), catalase (C) in rat heart after acute, chronic exercise and after different regimes of intermittent hypoxic training. Values are means \pm SE of 10 animals. Groups: 1 – control; 2 – acute exercise (30 min); 3 – chronic exercise (4 wk); 4 – intermittent hypoxic training (IHT) (2 wk); 5 – chronic exercise (4 wk) + IHT during the first two weeks; 6 – chronic exercise (4 wk) + IHT during the last two weeks. * – Significant differences from control ($p < 0.05$); # – significant differences from chronic exercise rats ($p < 0.05$) (Student's *t*-test)

Table I

State of glutathione system and activity of glucose-6-phosphate dehydrogenase in rat heart after acute and chronic exercise, and after different regimes of intermittent hypoxic training

Groups	GSH μmol/mg protein	GR nmol NADPH/ min/mg protein	GPx μmol/GSH/ mg protein	G6PDH nmol NADPH/ min/ mg protein
Control	1.70±0.10	8.22±0.64	7.42±0.86	2.79±0.18
Acute exercise (30 min)	1.0 ±0.07**	7.06±0.88	5.27±0.59*	3.14±0.16
Chronic exercise (4 wk)	2.02±0.14*	10.45±1.06*	8.24±1.01	3.51±0.21*
IHT (2 wk)	2.50±0.20**	9.13±0.82	11.29±1.12**	3.04±0.26
Chronic exercise (4 wk) + IHT during the first two weeks	2.44±0.16**,#	9.79±1.10	10.84±0.96*,#	3.18±0.41
Chronic exercise (4 wk) + IHT during the last two weeks	2.18±0.12**	10.0±1.12	10.79±0.89*,#	3.27±0.18*

Values are means ± SEM. n = 10 in each group

Significance: * – p<0.05 vs control; ** – p<0.01 vs control; # – p<0.05 vs chronic exercise (Student's *t*-test)
GSH – reduced glutathione; GR – glutathione reductase; GPx – glutathione peroxidase; G6PDH – glucose-6-phosphate dehydrogenase; IHT – intermittent hypoxic training

Discussion

In the present study, the acute high-intensity exercise induced an increase in TBARS level and decrease in GSH content as well as SOD, GPx, and GR activities in heart. These data support the hypothesis of oxidative stress development in tissues following intracellular antioxidant disorders at acute exercise (2, 22, 35). We can suppose that this is a result of compensatory mechanism directed against the enhanced production of ROS during strenuous exercise (7, 22).

Despite extensive research over the years, the relationship between free radical generation, antioxidant defense system and exercise in heart remains controversial (17, 22, 26). The discrepancies may be related to differences in exercise mode, intensity, and duration of training program. It have been reported that endurance training may reduce lipid peroxidation (LPO) in heart (17, 19), increase the level of antioxidants and GSH-related enzymes (22, 29). At the same time, some authors indicated that chronic exercise had no effect or it decreased activities of total superoxide dismutase, catalase (13, 17) and GSSG reductase and it enhanced thioredoxin reductase (17, 33) in rat myocardium. Nakao et al. (26) showed that endurance-swimming program (1 h/day, 5 days/wk, for 6 wk) diminished CuZn-SOD activity, but increased protein level and mRNA abundance in mouse heart. Leeuwenburgh et al. (19) observed a significant decrease of myocardial GSH content and total glutathione in rats undergoing rigorous

swim training, up to 6 h/day for 4 weeks. However, in moderately swim-trained rats, myocardial GSH content was maintained or increased slightly. Somani et al. (35) reported that in rat heart, acute exercise resulted in a larger increase in antioxidant enzymes activities than chronic exercise.

After swim training, when rats had high-intensity exercise daily during 4 weeks, TBARS content in rat heart remained higher than the control level (Fig. 1A). We used long-term high-intensity exercises that have larger radical generation and tissue damage (2). Moreover, another potential mechanism involved in the oxidative stress response to swimming exercise could possibly be the redistribution of the blood flow, that is, elevated blood flow in heart, lung, and red gastrocnemius muscle, leading to increased mitochondrial respiration, which results in an increase production of ROS (26).

In our studies, we showed that an endurance-swimming program did not increase levels of enzymatic antioxidants and the protection was based on an elevation of the concentration of GSH in myocardium and increase in GR activity (Fig. 1B, C). This may be explained by the activation of NADPH-supplying enzyme glucose-6-phosphate dehydrogenase (by 26%; $p < 0.05$) to maintain intracellular reduced glutathione stores. Our results indicated that endurance-trained rats improved their ability to keep tissue glutathione redox status in comparison with untrained animals (Table I).

The results of this study are in accordance with the opinion of some researchers (15, 34) that chronic exercise has dual effect: long-term exercise causes an oxidant formation and, perhaps as consequence, induces antioxidant synthesis that restricts the effects of oxidants. Oxidative stress is indicated by the accumulation of TBARS content in heart of chronically exercised animals (Fig. 1A), therefore we used sessions of IHT for correction of such conditions.

IHT is defined as repeated short-term hypoxia with normoxic intervals (5). Acute hypoxia and especially subsequent reoxygenation induce excessive ROS generation that is typical for hypoxia-reoxygenation and ischemia-reperfusion in a variety of organs (20). It is considered that repeated moderate oxidative stress in hypoxia-reoxygenation episodes is an important factor in training of antiradical defense systems (12). Vanden Hoek et al. (38) observed that mitochondrial ROS generation could activate signaling cascades involved in protective responses of cardiomyocytes. Some authors indicated, that recurrent hypoxia-reoxygenation exposure attenuates ROS formation in heart muscle, hepatocytes, brain neurons (20, 21, 38). In contrast, our results showed that proposed regimen of IHT caused some increase in TBARS content in myocardium in comparison with normoxic rats (Fig. 1A). This may be explained by the longer hypoxic exposure we used in our experiments. It is known that severity of hypoxic injury depends on the duration of hypoxic exposure (20).

Changes in antioxidant defense systems induced by intermittent hypoxia were demonstrated both in animal experiments and in studies in human. In rat brain, IHT resulted in an increase in SOD activity and decrease in Fe/ascorbate induced LPO (12). In contrast, Sazontova et al. (31) indicated that adaptation to interval hypoxia did not induce activation of myocardial catalase and SOD. It was shown that in red cells normobaric IHT (inhalation of gas mixture containing 10% O₂ in regime – 5 min

hypoxia and 3 min normoxia for 90 min) did not affect the catalase activity and it increased SOD activity by 25%. In liver *in vitro*, the intensity of LPO was decreased with the persisting activities of catalase and SOD (30). Normobaric IHT prevented the possibility of LPO activation, regulated energy metabolism, increased activity of antioxidant enzymes in blood serum of patients with bronchial asthma, ischemic heart disease, moderate hypertension (18). In agreement with these data, we found elevations in GSH content and in the activity of SOD, CAT, GPx in rat heart after intermittent hypoxic training (Fig. 1B, C; Table I). We can suppose that oxidative stress evoked by hypoxia-reoxygenation might trigger adaptations of antioxidative enzymes. The increased level of the antioxidant defense system leads to the scavenging of excess free radicals and, thereby, it may contribute to the decrease of oxidative damage under such conditions. The lack of major changes in the activation of G6PDH is consistent with maintenance of normal aerobic metabolism during intermittent hypoxia by increased in oxidative enzyme activity and mitochondrial density (9, 36). Different combinations of IHT with swimming training induced a dramatical diminution in TBARS content and increase in SOD, CAT and GSH-related enzymes activities in heart in comparison with chronically exercised rats alone (Fig. 1A, B, C). We consider that a tendency to G6PDH activity increase in heart promotes to maintenance the intracellular GSH recycle in reducing state. Although, the *de novo* synthesis of GSH and the enzyme activity of the γ -glutamyl cycle in these processes are also important (33), without a direct measurement this hypothesis remains speculative.

It is known that the ROS act as signaling molecules and the changes in redox state due to repetitive hypoxia-reoxygenation during IHT may stimulate expression of transcription factors such as the hypoxia-inducible factor-1 (HIF-1) (39). Transactivation of HIF-1 is necessary for the induction of several genes, that encode erythropoietin, glycolytic enzymes, VEGF, protective protein synthesis including enzymes of the antioxidative defense system (9, 10, 39, 40). Unfortunately, very little is known about how IH influences gene expression in heart. An attractive hypothesis concerning a potential pathway of adaptation is that intermittent hypoxic stimuli induce a continuous state of preconditioning, protecting the heart from subsequent exposures to chronic exercise. It is considered that such mediators as ROS play important roles in preconditioning and they may act synchronously by the activation of protein kinase C pathways, upregulation of antioxidant defenses, stress proteins, the activation of mitochondrial K-ATP channels (39, 40).

In summary, the present study demonstrated that sessions of IHT had protective effect against oxidative stress in heart evoked by high-intensity chronic exercise. This influence was proven by the data obtained concerning the prevention of lipid peroxidation during prolonged swimming, and the increase of GSH content, activities of SOD, CAT and GPx.

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