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Activity and Expression of the Antioxidant Enzyme MnSOD in the Mitochondria During Prolonged Hypoxia/hyperoxia Exposure

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ABSTRACT

The purpose of this study was to determine the influence of repetitive moderate hypoxia and hyperoxia (H/H) on the specific activity of manganese superoxide dismutase (MnSOD), MnSOD protein synthesis and mRNA MnSOD expression as well as on the pro-/antioxidant homeostasis in lung and heart mitochondria of rats exposed to acute severe hypoxia. It was shown that H/H pretreatment 5 cycles of 5 min hypoxia (10% O_2 in N_2) alternated with 5 min hyperoxia (30% O_2 in N_2) daily for two weeks reduced the acute hypoxia-induced lipid peroxidation, increased the GSH/GSSG ratio, the level of GSH and the activity of glutathione peroxidase in lung and heart mitochondria. In lung mitochondria H/H training resulted in increases in both the activity of MnSOD and protein synthesis. In heart mitochondria adaptation to moderate H/H enhanced the MnSOD protein expression and decreased MnSOD the acute hypoxia-induced hyperactivation. At the same time, in mitochondria of both tissues MnSOD mRNA expression was lower than in mitochondria normoxic rats. This study indicates that the increased level of MnSOD-antioxidative defense as well as the maintenance of glutathione redox status in mitochondria from H/H-treated rats is of the fundamental importance for the oxidative stress keeping at the tolerable level and confirms that mitochondrial protection during H/H may be mediated through the modulation of mitochondrial antioxidant levels.

Key words: Hypoxia/hyperoxia, mitochondria, MnSOD activity, MnSOD protein and mRNA expression, glutathione system

INTRODUCTION

Mitochondria, as a consequence of their biological functions, are always exposed to production of Reactive Oxygen Species (ROS) and contain an elaborate antioxidant defense system to counteract it and to prevent oxidative stress (Jezek and Hlavata, 2005). Manganese superoxide dismutase (MnSOD) is the first in a series of protective antioxidant enzymes that scavenge toxic superoxide radicals generated by the mitochondrial electron transport chain. This enzyme catalyzes the dismutation of O_2-to molecular oxygen and hydrogen peroxide and is insensitive to cyanide and H_2O_2. MnSOD is an inducible homotetrameric protein (96 kDa) which is synthesized in the cytoplasm as a precursor and transported post-translationally into the mitochondrial matrix via an amino-terminal targeting sequence (Ohno *et al*., 1994).

The down-regulation of MnSOD increased susceptibility to oxidative stress and caused severe mitochondrial dysfunction resulting from elevation of ROS. Numerous studies have shown that

MnSOD can be induced to protect against peroxidant insults resulting from cytokine treatment, irradiation, certain tumors and ischemia/reperfusion. In addition, overexpression of MnSOD has been shown to protect against pro-apoptotic stimuli as well as ischemic damage (Liu *et al*., 2004; Shan *et al*., 2007).

The expression and activity of MnSOD may be altered under several physiological and pathophysiological conditions including hypoxia-reperfusion and hyperoxia. For example, MnSOD is particularly responsive to and upregulated by oxidative stress (Macmillan-Crow and Cruthirds, 2001), consequently, MnSOD protein level and activity may play a critical role in adaptation to different levels of oxygen and may be an important adaptive value.

It was reported earlier that intermittent hypoxia and hyperoxia might effectively stimulate various metabolic processes and this phenomenon widely used in sport and medical practice (Clanton and Klawitter, 2001). The combined training including hypoxic and hyperoxic episodes provides stable adaptive protection in patients suffering from coronary heart disease and induces significant changes in the human blood lipid spectrum and energy metabolism (Maslov *et al*., 2004). The brief periods of hypoxia and hyperoxia improved resistance of heart, liver and brain membrane structures in rats to severe hypoxia and exhaustive exercise (Sazontova and Arhipenko, 2009). Many researchers explained the significant role of MnSOD in the prevention of oxidative stress (Shan *et al*., 2007; Gonchar and Mankovska, 2010). However, little is known about the participation of MnSOD expression in the formation of compensatory-adaptive responses to intermittent hypoxia/hyperoxia. To estimate the effectiveness of hypoxia/hyperoxia adaptation we used acute hypoxic influence.

The purpose of this study was to determine the influence of repetitive moderate hypoxia/hyperoxia on the MnSOD specific activity, MnSOD protein synthesis and mRNA MnSOD expression as well as on the pro-/antioxidant homeostasis in lung and heart mitochondria of rats exposed to acute severe hypoxia.

MATERIALS AND METHODS

Chemicals: All chemicals were purchased from Sigma, Fluka and Merck and were of the highest purity.

Animals and experimental procedure: Male Wistar rats weighing 220-260 g were used. The experimental animals were housed in Plexiglas cages (4 rats^{-1}) and kept in an air-filtered and temperature-controlled (20-22°C) room. Rats received a standard pellet diet and water *ad libitum* and were kept under artificial light-dark cycle of 12 h. The present study was approved by the Animal Ethics Committee at the Bogomoletz Institute of Physiology, Kyiv, Ukraine (Protocol No. 5/17). Rats were randomly divided into four groups (eight animals in each). Animals of group I were kept under normoxic conditions and served as a control. In group II, rats were exposed to a single action of acute hypoxia by breathing with the hypoxic gaseous mixture (7% O_2 in N_2) for 60 min. Group III included animals subjected to sessions of intermittent hypoxia-hyperoxia. We applied repeated short-term 5-min inhalation of gaseous mixture containing 10% O_2 in N_2 with 5-min intervals of hyperoxia (breathing with the hyperoxic gaseous mixture containing 30% O_2 in N_2) under normobaric condition in a special chamber where the temperature and humidity were maintained at 21-26°C and 55-60%, respectively. Rats had five such sessions daily for 14 days. The gaseous mixtures with low and high content of O_2 were obtained with the help of a device (Metaks Co) operating on the membrane gas partition principle with on-line computer control of O_2 levels

in isolated animal cage. Rats of group IV were exposed to acute hypoxia on the first day after cessation of the intermittent hypoxia-hyperoxia training course. Animals were decapitated immediately after the experiment. At the time of sacrifice, the rats were lightly anesthetized with ether.

Biochemical assays: The lung and heart mitochondria were isolated by differential centrifugation as described by Fisher *et al.* (1973) and Mela and Seitz, 1979. The mitochondrial preparations were analyzed after solubilization in 0.5% deoxycholate for 60 min at 0-4°C.

Lipid peroxidation (LPO) in isolated mitochondria was measured from the formation of thiobarbituric acid-reactive substances (TBARS) using the method of Buege and Aust (1978). Manganese superoxide dismutase (MnSOD) (EC 1.15.1.1) activity was measured by the method of Misra and Fridovich (1972), which is based on the inhibition of autooxidation of adrenaline to adrenochrome by SOD contained in the examined samples. The samples were preincubated at O°C for 60 min with 6 mM KCN which produces total inhibition of Cu, Zn-SOD activity. Activity of selenium-dependent glutathione peroxidase (GPx) (EC 1.11.1.9) was estimated by the methods of Rotruck *et al.* (1973). Reduced glutathione (GSH) and oxidized glutathione (GSSG) were measured by the method of Anderson (1985). Mitochondrial protein concentration was estimated by the Lowry method, using bovine serum albumin as a standard.

Protein level determination of MnSOD by Western Blot: Isolated mitochondrial protein extracts (100 μg) were separated on SDS-polyacrylamide gel (12%) according to Laemmli (1970) and transferred to a polyvinylidene fluoride membranes by semi-dry electrophoretic transfer. The membranes were then blocked with 5% nonfat dry milk in Tris Buffered Saline Tween-20 (TBST) buffer (50 mM Tris-HCI, 150 mM L^{-1} NaCI and 0.1% Tween, pH 7.4) for 1 h at 37°C . MnSOD proteins were detected using primary monoclonal antibody for SOD-2 (Sigma-Aldrich Co) at a dilution 1:1000 for 2 h at 37°C, followed by incubation with horseradish peroxidase-conjugated secondary antibody (Sigma-Aldrich Co.) (1:2000) for 1 h at 37°C. Each antigen-antibody complex was visualized by amino-ethylcarbazol reaction. The MnSOD band intensities were quantified by densitometry with a computerized image processing system (GelPro Analyzer). Results were expressed as percentages of control values.

RNA extraction and RT-PCR for MnSOD analysis: Total RNA was isolated using a commercial kit Trizol *RNA* Prep100 (Isogen, Russia) according to the manufacturer's instruction. Reverse Transcription (RT) was performed using RevertAid™ H Minus First Strand cDNA synthesis kit (Fermentas, Lithuania), 1.2-1.5 μg of total RNA and Random hexamer primers. Obtained one-strand cDNA was used for real-time PCR. PCR primers for MnSOD and glyceraldehyde-3-phosphatedhydrogenase (GAPDH) were purchased from "Fermentas" (Lithuania) and contained the following sequences: MnSOD sense primer: 5′-CTGAGGAGAGCAGCGGTCGT-3′, MnSOD antisense primer: 5′- CTTGGCCAGCGCCTCGTGGT-3′; GAPDH sense primer: 5′-GGGTGTGAACCACGAGAAAATATGA-3′, GAPDH antisense primer: 5′-AGCACCAGTGGATGCAGGGGATGAT-3′. Mixture for amplification contained 5 μL of 5x PCR buffer, 1.5 mM magnesium sulphate, 0.2 mM of each dNTP, 3 μL cDNA, 1U Tag-polymerase (AmpliSens, Russia), 30 pM of each of the primers and deionized water to 25 μL of total volume. The mixture was subjected to 40 cycles for MnSOD and 30 cycles for GAPDH of sequential steps in an automated thermal cycler "Applied Biosystems 2700" (Perkin Elmer, USA):

denaturation (1 min at 94°C for MnSOD and GAPDH), annealing (40 sec at 61°C for MnSOD and 50 sec at 65.5°C for GAPDH), then elongation (1 min at 72°C for MnSOD and GAPDH). This program was complete with a final extension for 7 min at 72°C. After amplification, the products were separated by electrophoresis on 1.6% agarose gels and ethidium bromide-stained bands were visualized by UV transillumination (BioCom, Russia), the fluorescence intensity was quantified using a Gel Doc 2000 system (BioRad). There were no significant differences in intensity of GAPDH levels between experimental groups.

Statistical analysis: Data are expressed as Means±SEM for each group. The differences among experimental groups were detected by one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test.

RESULTS

In lung and heart mitochondria extracted from rats exposed to acute severe hypoxia the level of LPO increased significantly by 54 and 33% ($p<0.05$), respectively, as compared to the normoxia (Fig.1a). Simultaneously we registered a decrease in the GSH concentration and GSH/GSSG ratio (Table 1). Acute hypoxia also resulted in a reduction of the activity of GPx in lung and heart mitochondria by 20 and 44% ($p<0.05$), respectively (Table 1). In lung mitochondria, the MnSOD activity (Fig. 1b) and MnSOD protein content (Fig. 2a and b) decreased by 21 and 19% ($p<0.05$), respectively, meanwhile MnSOD mRNA level was increased by 26% ($p<0.05$) in comparison with control (Fig. 3). In heart mitochondria MnSOD activity and protein content increased by 38 and 14% ($p<0.05$), respectively, while the MnSOD mRNA level demonstrated a 1,3-fold decline as compared with the control. After sessions of intermittent hypoxia/hyperoxia, the TBARS concentration in heart mitochondria did not exceed the control level, in lung mitochondria this index of LPO was significantly higher than in normoxic rats ($p<0.05$) (Fig.1a, b). In mitochondria of both tissues GSH concentration and GSH/GSSG ratio remained on control level (Table 1). In heart mitochondria, we observed no significant changes in the GPx activity. At the same time, the activity of this enzyme in lung mitochondria demonstrated a trend toward decrease.

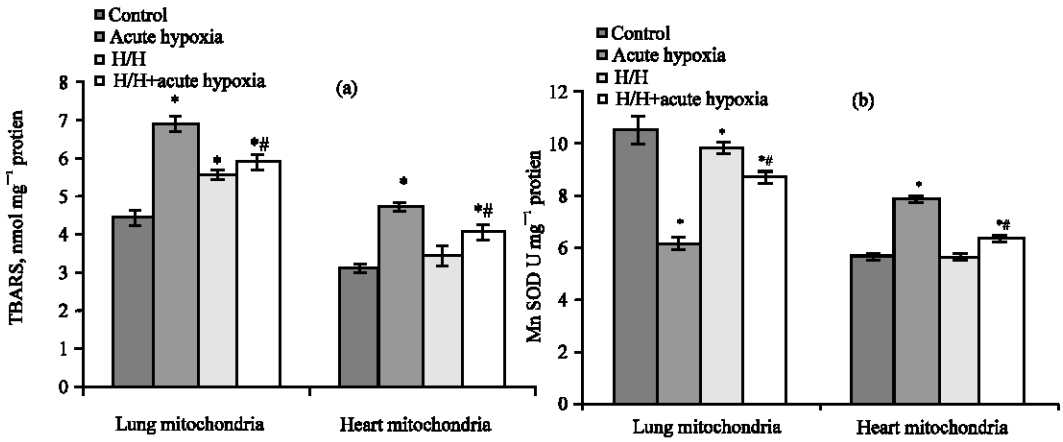

Fig. 1(a, b): Effect of intermittent hypoxia/hyperoxia (H/H) and acute hypoxia on mitochondrial lipid peroxidation (a) and MnSOD activity (b). Values are mean ± SEM. n = 8 in each group. The data were analyzed for statistical significance using ANOVA followed by Bonferroni posthoc test. *$p<0.05$ vs. control; #$p<0.05$ vs. acute hypoxia (group 2)

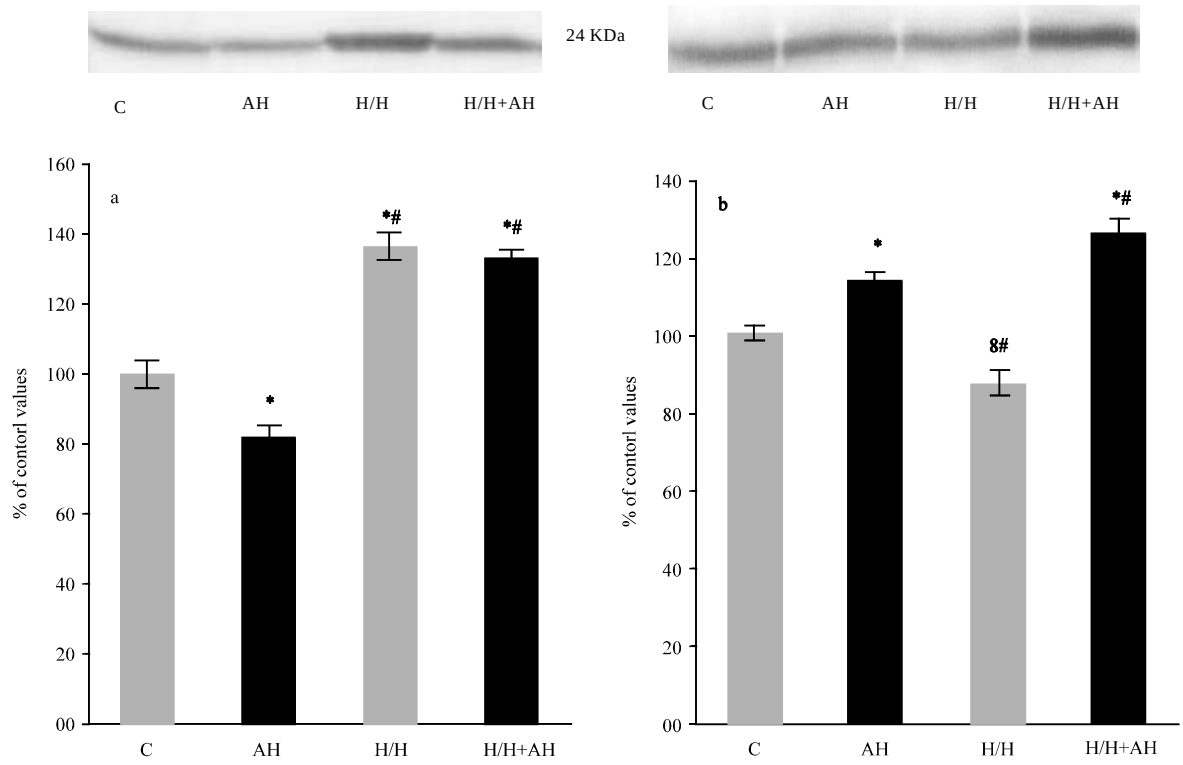

Fig. 2(a-b): Effect of intermittent hypoxia/hyperoxia and acute hypoxia on MnSOD protein expression in lung mitochondria (a) and in heart mitochondria (b). Representative Western blot and densitometric analysis of MnSOD. Isolated mitochondrial protein extracts were separated by performing SDS-PAGE and subsequently electroblotted onto PVDF membranes. The blot was probed using a monoclonal antibody against MnSOD. Final Western blot figured as histogram is expressed as mean percentages (±SD) over control values from three independent experiments. Statistically significant differences are indicated as $^{*}p<0.05$ vs. control; $^{\#}p<0.05$ vs. acute hypoxia

H/H training led to an enhancement of the MnSOD protein expression by 36% ($p<0.05$) and diminution in MnSOD mRNA level by 16% ($p<0.05$) in lung mitochondria (Fig. 3). In heart mitochondria from trained rats, the MnSOD protein content was decreased by 13% ($p<0.05$) in comparison with control and MnSOD mRNA was remained on control level. Acute severe hypoxia after cessation of intermittent hypoxia/hyperoxia resulted in decreases in LPO level in lung and heart mitochondria by 14 and 17% ($p<0.05$) as compared with untrained rats (group II) (Fig. 1A, B). In rats of group IV mitochondrial actives of MnSOD was higher in lung and lower in heart mitochondria than those in group II rats ($p<0.05$). However, the GPx activity remained at the level observed in rats exposed to acute hypoxia but was smaller than that in control rats (Table 1). In both type of mitochondria the exposure of H/H training animals to acute hypoxia resulted in a significant increase in MnSOD protein synthesis ($p<0.05$) and a decrease in MnSOD mRNA expression ($p<0.05$) in comparison with the control (Fig. 3). In this rat group changes in glutathione pool were following: GSH level and GSH/GSSG ratio were increased compared to group II ($p<0.05$) (Table 1).

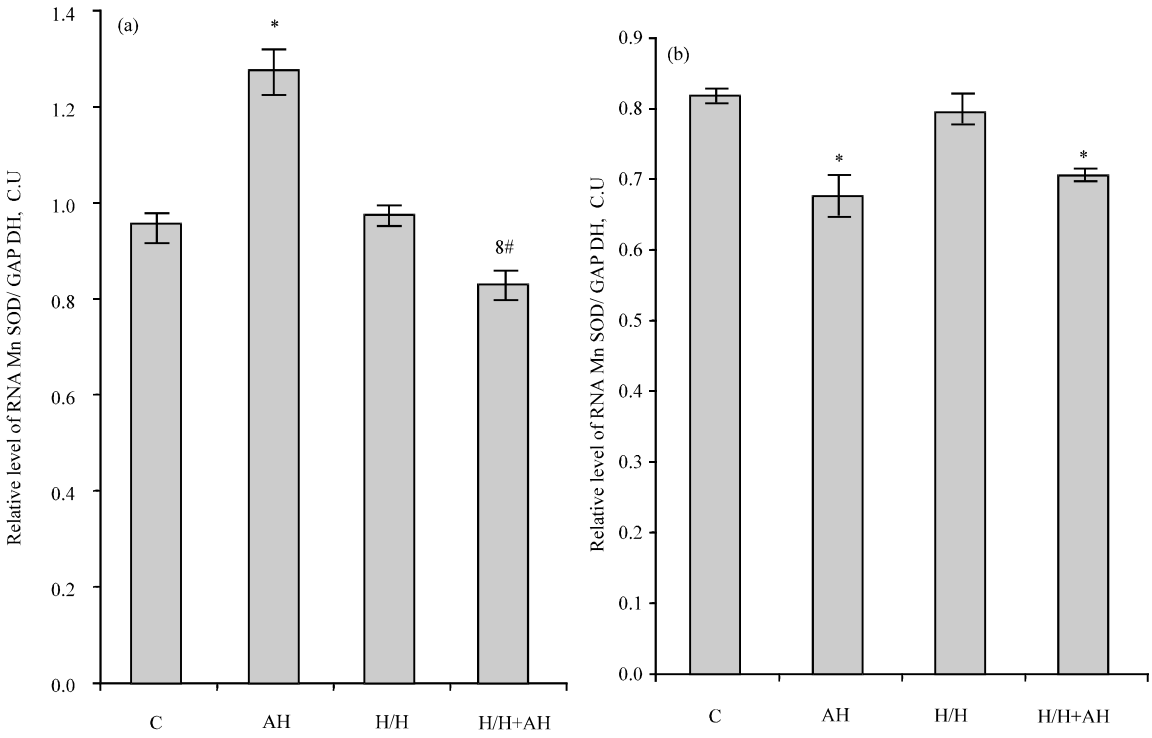

Fig. 3a, b: RT-PCR analyses of lung (a) and heart (b) MnSOD mRNA levels after intermittent hypoxia/hyperoxia and acute hypoxia. Densitometric values corresponding to the levels of MnSOD mRNA were normalized to glyceraldehyde-3-phosphatedehydrogenase (GAPDH) mRNA as the internal standard. Final data were obtained from four separate experiments. Statistically significant differences are indicated as $^{*}p<0.05$ vs. control; $^{\#}p<0.05$ vs. acute hypoxia

Table 1: Changes in mitochondrial reduced glutathione content and glutathione peroxidase activity after acute hypoxia and intermittent hypoxia/hyperoxia (H/H)

Groups	Experimental conditions	Lung mitochondria	Heart mitochondria
	Reduced glutathione (nmol mg^{-1} prot.)		
Control		3.92±0.05	3.16±0.08
Acute hypoxia		3.33±0.07*	2.56±0.03 *
H/H		3.85±0.06	3.22±0.09
H/H + acute hypoxia		3.75±0.02 *#	3.06±0.04#
	GSH / GSSG		
Control		10.60±0.15	14.36±0.65
Acute hypoxia		6.16±0.27 *	9.14±0.95 *
H/H		9.87±0.19	14.00±0.10
H/H + acute hypoxia		8.72±0.18 *#	12.88±0.87 #
	Glutathione peroxidase (µmol GSH min^{-1} mg^{-1} prot)		
Control		8.30±0.33	5.61±0.25
Acute hypoxia		6.65±0.27 *	3.90±0.18 *
H/H		7.80±0.56	5.92±0.35 #
H/H + acute hypoxia		7.17±0.14 *#	4.96±0.33 *#

Values are Means±SEM, n=8. *p<0.05 vs. control; #p<0.05 vs. acute hypoxia

DISCUSSION

In this study we demonstrated that in lung and heart mitochondria MnSOD reacts differently to the action of acute stress. In lung mitochondria MnSOD, one of the most important antioxidant components of a cell, was inactivated after severe hypoxia. MnSOD constitutes of about 10-15% of the total SODs and is localized in the mitochondria of type II pneumocytes, alveolar macrophages and bronchial epithelium cells of human lungs. MnSOD mRNA is appreciably expressed in the cells in airway walls, the septal tips of alveolar dusts and also in the arteriolar walls located adjacent of airways (Tsan, 2001). In the present experiments, it was found decreases in the MnSOD activity and in MnSOD protein content. At the same time the MnSOD mRNA expression was enhanced by 35% ($p<0.05$) in comparison with normoxia. These results are in good agreement with the findings that hypoxia decreases both the MnSOD activity and protein expression in rabbit (Russell and Jackson, 1993) and mice (Russell *et al*., 1995) lung. In contrast, the cultured alveolar type II epithelial cells and lung fibroblasts showed decreased MnSOD gene expression after *in vitro* exposure to 2.5% O_2 (Jackson *et al*.,1996). As was described previously, oxidants and cytokines generally cause the MnSOD induction (Warner *et al*., 1996). However, the elevation of transcription, coupled with the corresponding increased translation did not result in the stimulation of SOD activity. Mitochondrial-generated RNS and ROS can mediate post translational modifications of mitochondrial proteins that result in MnSOD inactivation by peroxynitrite (MacMillan-Crow *et al*., 1998).

At the same time, the concentration of MnSOD in heart mitochondria subjected to severe hypoxia was increased significantly in comparison with the control. This can be explained by a compensatory increase in the activity of this enzyme in response to the increased superoxide anion production which is known serves as a substrate for MnSOD. We also found that, in addition to the acute hypoxia-induced rise in enzymatic activity, the expression of MnSOD protein increased by 14% ($p<0.05$) against the background of the decreased expression of mRNA by 22% ($p<0.05$) which is indicative of the activation of synthesis of this enzyme. It seems probable that the induction of MnSOD under such conditions can be considered a defensive reaction to the excess production of active oxygen metabolites which, in turn, can activate the expression of antioxidant enzymes (in particular MnSOD) via different signal pathways. Similar increases in the activity and expression of MnSOD protein in response to the development of oxidative stress of different genesises were also reported by other researchers (Shan *et al*., 2007; Balkova *et al*., 2011).

In this study, we have shown that acute severe hypoxia triggers a series of events in lung mitochondria including a significant increase in basal LPO level as well as a decrease in the GSH/GSSG ratio which are essential indicators of oxidative stress in cell compartments (Dickinson and Forman, 2002; Gonchar and Mankovskaya, 2009). Oxidative stress resulting from excessive ROS production may be one of the major reasons for mitochondrial dysfunction and accumulation of mitochondrial damage (Rahman and MacNee, 2000).

Severe hypoxia may lead to an imbalance in the antioxidant status of the cells. Our experimental data showed that a decrease in the content of reduced glutathione as well as in activity of GPx that can result in accumulation of H_2O_2 which not only changes the mitochondrial redox status but can also participate in the Fenton reaction, leading to the production of noxious hydroxyl radicals. It was reported earlier that severe hypoxia depletes the GSH stores in liver mitochondria and leads to a short-term fall in the intracellular GSH levels in rat endothelial and alveolar cells under both *in vitro* and *in vivo* conditions (Mansfield *et al*., 2004). Mitochondrial GSH may also be susceptible to the hypoxia-induced oxidative stress imposed by TNF-α TNF-α is known

to deplete the cytosolic GSH levels transiently in lung epithelial cells. This depletion by TNF-α is thought to be due to oxidative stress from mitochondrial generation of $O2^-$ in the electron transport chain (Rahman *et al*., 1999).

For correction of the hypoxia-induced mitochondrial dysfunction, we used adaptive training where sessions of hypoxia were alternated with sessions of moderate hyperoxia. In the present study, we demonstrated that in lung mitochondria the proposed training regime exerts no effect on the GSH content, while GPx activity has a trend toward a decrease.

In lung mitochondria, exposed to prolonged H/H, RT- PCR revealed no changes in MnSOD mRNA level compared to the control. In contrast, the MnSOD activity and MnSOD protein level were upregulated. However, these changes occurred against the background of an increase in the TBARS level which is an indicator of some imbalance in pro-/antioxidant homeostasis in lung mitochondria. The excess of MnSOD protein expression without a concomitant increase in the level of GPx could potentially cause the accumulation of H_2O_2. This fact and also the enhancement in LPO stimulated *in vitro* during the H/H sessions suggest that lung mitochondria are sensitive to prolonged hypoxia and hyperoxia. In heart mitochondria H/H caused no change in activity and mRNA level of MnSOD, but decreased the protein expression of this enzyme in comparison with control.

In the present study, there was observed that H/H pretreatment significantly lowered the hypoxia-induced lipid peroxidation in lung as well as in heart mitochondria. The increase in the GSH/GSSG ratio in comparison with acute hypoxia is an indicator of the intensity of oxidative stress reducing in the mitochondria. The increase in GSH level and activities of GPx and MnSOD (in lung and decline hyperactivity of MnSOD in heart) in comparison with acute hypoxia suggest that mitochondrial protection during H/H may be mediated through the modulation of mitochondrial antioxidant levels. In both tissues acute hypoxia after H/H training caused an increase in MnSOD protein synthesis and a decrease in the MnSOD gene expression. The MnSOD mRNA levels do not necessarily correlate with protein expression or enzymatic activity because some factors might directly stimulated the SOD protein synthesis without transcriptional activation (Pardo and Tirosh, 2009). In our study, the subsequent elevation in MnSOD protein synthesis might be compensatory in response to oxidative stress and might results in a higher enzyme protein level. This explanation may be acceptable because mRNA usually have much shorter half-lives than enzyme proteins and can be degraded rapidly after transient upregulation (Sen and Packer, 1996).

Indeed, MnSOD is known as an inducible enzyme that may be activated in a variety of stressful conditions, including the changes in oxygen level (Shen *et al*., 2008). Thus, exposure to hyperoxia caused the enhancement of the MnSOD immunoreactivity especially in alveolar type II cells and in interstitial fibroblasts. Hypoxia/reoxygenation induced the expression of MnSOD mRNA in tissues of lung, kidney and liver tissues (Pardo and Tirosh, 2009; Shen *et al*., 2008). Under hypoxic condition, MnSOD gene expression might be regulated by redox-sensitive transcription factors, such as SP-1, AP-1 and NF-kB (Sen and Packer, 1996). Ohman *et al*. (1999) demonstrated that an increase in ROS can activate NF-kB, hence induce the transcription of regulated genes including MnSOD gene. In a number of studies it has been demonstrated that the MnSOD- inducing pathway in myocardial preconditioning involves ROS signaling (Shan *et al*., 2007; Haddad, 2002). The activity and expression of MnSOD were increased in a ROS-dependent manner in various forms of delayed preconditioning and its important protective role against ischemia/reperfusion injury has been well established (Shan *et al*., 2007; Balkova *et al*., 2011).

CONCLUSION

Therefore, the results reported here clearly demonstrate that H/H exerts different effects on the MnSOD activity, MnSOD protein level and MnSOD mRNA expression in lung and heart mitochondria which confirm the tissues specific adaptation to various oxygen levels. This study indicates that the increased level of MnSOD-antioxidative defense and the maintenance of glutathione redox status in the mitochondria from H/H-treated rats are of the fundamental importance for the oxidative stress keeping at the tolerable level and also for the formation of protective mechanisms against new oxidative stress.

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