N-acetylcysteine Treatment Prevents the Up-Regulation of MnSOD in Chronically Hypoxic Rat Hearts

P. BALKOVÁ1,3, M. HLAVÁČKOVÁ1, M. MILEROVÁ2,3, J. NECKÁŘ2,3, F. KOLÁŘ2,3, F. NOVÁK1,3, O. NOVÁKOVÁ1

1Department of Cell Biology, Faculty of Science, Charles University in Prague, 2Institute of Physiology, Academy of Sciences of the Czech Republic, Prague, 3Centre for Cardiovascular Research, Prague, Czech Republic

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Summary
Chronic intermittent hypoxia (CIH) is associated with increased production of reactive oxygen species that contributes to the adaptive mechanism underlying the improved myocardial ischemic tolerance. The aim was to find out whether the antioxidative enzyme manganese superoxide dismutase (MnSOD) can play a role in CIH-induced cardioprotection. Adult male Wistar rats were exposed to intermittent hypobaric hypoxia (7000 m, 8 h/day, 25 exposures) (n=14) or kept at normoxia (n=14). Half of the animals from each group received N-acetylcysteine (NAC, 100 mg/kg) daily before the hypoxic exposure. The activity and expression of MnSOD were increased by 66 % and 23 %, respectively, in the mitochondrial fraction of CIH hearts as compared with the normoxic group; these effects were suppressed by NAC treatment. The negative correlation between MnSOD activity and myocardial infarct size suggests that MnSOD can contribute to the improved ischemic tolerance of CIH hearts.

Key words
Chronic hypoxia • MnSOD • Ischemia/reperfusion • Myocardial infarction • Cardioprotection

Introduction
It has been shown that reactive oxygen species (ROS) formed under stress conditions, such as exercise, hyperthermia or various forms of preconditioning, may enhance the tolerance of the heart to ischemia-reperfusion injury by stimulation of cellular antioxidant defence systems that maintain optimal redox balance (Hoshida et al. 2002). The main source of ROS in myocardium is the respiratory chain of the inner mitochondrial membrane where manganese superoxide dismutase (MnSOD; SOD2), the key antioxidative enzyme, is also localized (Starkov et al. 2008). Several signalling pathways involving ROS as a second messenger in conjunction with the induction of MnSOD gene expression have been identified (Rogers et al. 2001, Hussain et al. 2004). MnSOD is an inducible homotetrameric protein, synthesized in the cytoplasm as a precursor and imported post-translationally in the mature form into the mitochondrial matrix (Wispe et al. 1989). MnSOD catalyzes the dismutation of two superoxide radicals, generating hydrogen peroxide and molecular oxygen (McCord et al. 1969). This enzyme is most likely of crucial importance for cell viability as mice lacking MnSOD die from dilated cardiomyopathy within the first days after the birth (Li et al. 1995). Adaptation to chronic intermittent hypoxia (CIH) is known to protect the heart against acute ischemia/reperfusion injury. The mechanism underlying induction of the long-lasting protected phenotype of CIH adapted hearts is not precisely understood (Kolář et
It has been proposed that ROS generated during the adaptation period are important components of a signalling pathway leading to this form of cardioprotection as chronic treatment of rats with an antioxidant eliminated the infarct size-limiting effect of CIH (Kolář et al. 2007). The main goal of our study was, therefore, to find out whether CIH affects the activity and expression of MnSOD in rat heart mitochondria and whether these changes depend on ROS production. Levels of pyruvate dehydrogenase (PDH) in mitochondrial and cytosolic fractions were measured as a marker of mitochondrial integrity. We showed that CIH led to the up-regulation and activation of MnSOD, and these effects were absent in animals chronically treated with the antioxidant N-acetylcysteine (NAC) during the adaptation period. Moreover, the negative correlation between MnSOD activity and myocardial infarct size suggests that this enzyme can contribute to the improved ischemic tolerance of CIH-adapted hearts.

Materials and Methods

Animal model

Adult male Wistar rats were exposed to intermittent hypobaric hypoxia of 7000 m for 8 h/day, 5 days a week, as described earlier (Kolář et al. 2007). Barometric pressure ($P_B$) was lowered stepwise, so that the final level equivalent to the altitude of 7000 m ($P_B=306.8$ mm Hg; $P_{O_2}=63.8$ mm Hg) was reached after 13 exposures; the total number of exposures was 25. The control group of animals was kept at normoxia for the same period of time. One-half of the rats from each group received NAC (dissolved in saline and neutralized with sodium hydroxide) subcutaneously in a dose of 100 mg/kg daily before each hypoxic exposure and the remaining rats received the same volume of saline (2 ml/kg) in the corresponding manner. Rats were fed standard laboratory diet and had free access to water. All rats were employed on the next day after the last hypoxic exposure and sacrificed by decapitation. Hearts were rapidly excised, washed in cold saline and left ventricular (LV) walls with the septum were dissected, weighed and used for analysis. The study was conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). The experimental protocol was approved by the Animal Care and Use Committee of the Institute of Physiology, Academy of Sciences of the Czech Republic.

Isolation of mitochondrial and cytosolic fractions

LV with septum were chopped by scissors in ice-cold medium (10 vol of medium to 1 vol of tissue) consisting of (in mM) 20 Tris-HCl, 250 sucrose, 2.5 EGTA, 5 EDTA, 100 NaF, 0.3 phenylmethylsulfonyl fluoride, 0.2 leupeptin, and 0.02 aprotinin (pH 7.4) and then homogenized by using a Potter-Elvehjem homogenizer with a loose-fit Teflon pestle. The homogenate was centrifuged at 800 $\times$ g for 20 min. The supernatant was centrifuged at 8,000 $\times$ g for 10 min. The mitochondrial pellet was washed (1 ml) twice and finally resuspended in the homogenization buffer. Supernatant was then centrifuged at 100,000 $\times$ g for 60 min to obtain the cytosolic fraction. All steps were performed at 4 °C. Samples for the measurement of MnSOD activity were sonicated using three 10-s bursts. Mitochondrial fraction for immunoblot analyses was extracted with 1 % Triton X-100 on ice for 60 min, the resulting detergent-treated supernatant was used. Triton X-100 was also added to the cytosolic fraction to reach the final concentration of 1 %. Protein content was determined according to Lowry modified by Peterson (Peterson et al. 1977). Neither hypoxia nor NAC influenced the protein yields in mitochondrial and cytosolic fractions (data not shown).

SDS-PAGE and Western blot analysis

Mitochondrial extracts and cytosolic fractions were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis on 15 % bis-acrylamide gel at

| Table 1. Expression of common housekeeping proteins. |
|------------------------------------------|
|  | Normoxia | Hypoxia |
|---|----------|--------|
| Actin | 42 kDa  |        |
| $45.9\pm2.5$ | 54.3±2.6* |
| Calsequestrin | 55 kDa  |        |
| $43.9\pm3.2$ | 56.0±3.2* |
| GAPDH | 36 kDa  |        |
| $35.8\pm4.4$ | 64.1±4.4* |
| $\beta$-tubulin | 50 kDa  |        |
| $43.4\pm3.0$ | 56.8±3.2* |

Representative Western blots are shown. Values are means ± S.E.M. from 6 hearts in each group (arbitrary units). *$P<0.05$ vs. normoxic group.
30 mA/gel for 90 min on a Mini-Protean III apparatus (Bio-Rad, Hercules, CA); the resolved proteins were transferred to a nitrocellulose membrane (Amersham Biosciences, Germany). After blocking with 5% dry low-fat milk in Tris-buffered saline with Tween 20 (TTBS) for 60 min at room temperature, membranes were washed and probed with rabbit anti-MnSOD polyclonal antibody (Stressgen Bioreagents, Victoria, Canada) and mouse monoclonal antibody to PDH E1β subunit (Molecular Probes, Oregon, USA). The membranes were washed again and incubated with the secondary swine anti-rabbit and anti-mouse (Sevapharma, Prague, Czech Republic; 1:4,000 in TTBS) antibodies, respectively, labelled with horseradish peroxidase for 60 min at room temperature. To ensure the specificity of immunoreactive protein, MnSOD standard (Stressgen Bioreagents, Victoria, Canada) were used. The samples from each experimental group were run on the same gel and quantified on the same membrane. The analysis of each heart sample was repeated at least three times and normalized to total protein.

For comparative quantification of the amount of monitored protein in Western blot analysis, an appropriate housekeeping protein as an internal standard in chronic experiments should always be carefully validated.

Measurement of MnSOD activity
Total superoxide dismutase (SOD) activity was determined by the modified nitroblue tetrazolium method (Elstner et al. 1983, Spitz et al. 1989) based on the generation of superoxide radicals from xanthine oxidase reaction that react further with nitroblue tetrazolium dye. Reduction of nitroblue tetrazolium by superoxide anion was measured spectrophotometrically at 540 nm (28 °C). Chloroform-ethanol extracts were then used to determine the SOD activity. The assay contained the following reagents: 0.1 mM phosphate buffer (pH 7.8), 4 mg/ml bovine serum albumin, 2 mg/ml nitroblue tetrazolium, and 1 mM xanthin. MnSOD activity was measured in the presence of 5 mM NaCN, the selective inhibitor of copper-zinc SOD.

Statistical analysis
The results are expressed as means ± S.E.M. Two-way ANOVA and subsequent Student-Newman-Keuls test were used for comparison of differences in parametric variables between the groups. Differences were assumed as statistically significant when $P<0.05$.

Results
Weight parameters
Adaptation of rats to CIH led to body growth retardation, pronounced hypertrophy of the right ventricle and moderate hypertrophy of the left ventricle. Treatment with NAC had no effect on body and heart weight parameters (Table 2).
Fig. 1. Level of MnSOD in homogenate (A), mitochondrial and cytosolic fractions (B) and enzyme activity in the mitochondrial fraction (C) from the myocardium of control (Cont) and N-acetylcysteine-treated (NAC) rats adapted to CIH and of normoxic animals. Sample loading was normalized to equal protein concentration in individual samples (homogenate 5 μg, cytosol 15 μg, mitochondria 5 μg). Representative Western blots are shown. For details see Materials and Methods. Values are means ± S.E.M. from 6 hearts in each group. *P<0.05 vs. corresponding normoxic group, †P<0.05 vs. corresponding untreated group.

Activity and expression of MnSOD

Neither CIH nor NAC treatment influenced the protein abundance of MnSOD in myocardial homogenate (Fig. 1A). On the other hand, both the activity and protein abundance of the enzyme were increased in the mitochondrial fraction of CIH hearts compared with the normoxic values (Fig. 1B, C). These effects of CIH were prevented by NAC. Interestingly, NAC treatment increased the MnSOD activity but not the expression in the mitochondrial fraction from normoxic tissue.

The low levels of MnSOD and PDH in cytosolic fractions from all experimental groups testify to good mitochondrial integrity. The protein abundance of PDH, as a mitochondrial marker, was decreased in homogenates of CIH hearts (Fig. 2A), but its abundance in the mitochondrial and cytosolic fractions was affected by neither CIH nor NAC treatment (Fig. 2B).

Correlation between MnSOD activity and infarct size

Relationship between the mean values of MnSOD activity in myocardial mitochondrial fraction and the mean infarct size normalized to the area at risk is presented in Fig. 3. Regression analysis demonstrated a negative linear relationship between MnSOD activity and infarct size with a correlation coefficient 0.93. Infarct size data were taken from our previous study (Kolář et al. 2007) using the same protocols of CIH and NAC treatment.

Discussion

In this study, we demonstrated that adaptation of adult rats to CIH significantly increased the activity and protein abundance of MnSOD in the mitochondrial fraction of LV myocardium but did not influence the expression of MnSOD in myocardial homogenate. This result is in line with our recent study which did not find any effect of CIH on the activity of MnSOD in myocardial homogenate (Kolář et al. 2007). The absence
of any effect on MnSOD in homogenate despite the significant up-regulation of the enzyme demonstrated in mitochondria can be possibly explained by a decrease in total mitochondrial mass caused by severe CIH. A decreased PDH level in the myocardial homogenate from CIH rats, shown in the present study, supports this view. Accordingly, Nouette-Gaulain et al. (2005) reported that CIH caused a global decrease in all OXPHOS complex activities in rat LV myocardium. Moreover, we showed previously that CIH reduced concentration of a mitochondrial inner membrane lipid marker cardiolipin in rat LV homogenate (Ježková et al. 2002).

The effects of CIH on MnSOD activity and protein abundance in the mitochondrial fraction were prevented by antioxidant NAC treatment during the adaptation period. These results conform to our recent observation that NAC attenuated the ROS-dependent cardioprotection induced by CIH (Kolář et al. 2007). However, at variance with our preliminary conclusion, the close negative correlation between MnSOD activity and myocardial infarct size suggests that this enzyme could possibly play a role in the improved mitochondrial ROS-buffering capacity and ischemic tolerance of chronically hypoxic hearts. Increased MnSOD activity was demonstrated also in the hearts of rats adapted to moderate hypoxia during early postnatal life (Zhu et al. 2004).

It is generally accepted that MnSOD represents the first line of cell defence against mitochondria-derived ROS and is involved in cardioprotection induced by...
induced by the hypoxic stimulus (Hoshida et al. 2002). The MnSOD-inducing pathway in myocardial preconditioning involves ROS signalling as demonstrated in a number of studies (Lecour et al. 2006, Chen et al. 2008). 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 (Yamashita et al. 1999). It has also been reported that heat-stress-induced preconditioning increased the expression of MnSOD mRNA in rats (Das et al. 1995) and total SOD activity in pig hearts (Liu et al. 1992). MnSOD elevation in the myocardium is supposed to be involved in the mechanism of cardioprotection induced by exercise (Hamilton et al. 2001). In agreement with these studies, Chen et al. (1998) demonstrated that overexpression of MnSOD in transgenic mice limited the infarct size.

Whereas numerous stimuli have been proposed to participate in the MnSOD up-regulation (Kiningham et al. 2004, Li et al. 1998, Oberley et al. 1987), no specific ROS-dependent transduction pathway leading to nuclear MnSOD gene induction has been described in the myocardium. Storz et al. (2005) suggested that the induction of the MnSOD gene with the subsequent increase in mitochondrial MnSOD expression in HeLa cells required transcription factor NF-xB initiated by ROS-activated protein kinase D. Other investigators demonstrated a dramatic elevation of mRNA and protein level of MnSOD induced by tumor necrosis factor-α and interleukin-1β in human skin fibroblasts and in mouse kidney, thymus, spleen and bone marrow tissues (Masuda et al. 1988, Wong et al. 1988). Interestingly, chronic hypoxia increased the level of pro-inflammatory cytokines in fetal guinea pig hearts (Oh et al. 2008) and in rat carotid bodies (Lam et al. 2008). PKC has been proposed as a further important player in the signaling pathway leading to MnSOD induction because PKC inhibitors prevented the up-regulation of MnSOD in the human adenocarcinoma cell line (Das et al. 1998). Accordingly, we observed that the induction of PKC-δ during the adaptation of rats to CIH was ROS-dependent because it was eliminated by NAC treatment (Kolář et al. 2007).

In the present study, NAC treatment completely prevented the increase in MnSOD activity and expression in mitochondria induced by CIH. It has also been reported that NAC abrogated the elevation of heat-stress protein 32 as another protective intracellular stress-responsive protein induced by the hypoxic stimulus (Borger et al. 1998). Heat-stress proteins in connection with MnSOD play a role in delayed cardiac preconditioning (Bolli et al. 2000). Suzuki et al. (2002) suggested that the rise in MnSOD activity is connected with the heat-stress protein 72-induced cardioprotection. In addition, heat-stress protein 72 acts as chaperone in the MnSOD maturation and its incorporation into mitochondria (Hoshida et al. 2002, Voos et al. 1993).

Interestingly, NAC treatment increased the activity of MnSOD in normoxic hearts without affecting its expression. Using the same experimental protocol, we observed a smaller infarct size in NAC-treated normoxic rats compared with untreated ones (Kolář et al. 2007). Menon et al. (2007) demonstrated that increased MnSOD activity was due to antioxidant properties of NAC. In addition, the restoration of MnSOD activity by NAC reduced oxidative stress and attenuated the development of myocardial dysfunction in diabetic rats (Xia et al. 2006). It appears likely that NAC acts as indirect antioxidant protecting MnSOD activity by preventing nitration of critical tyrosine residues in its active site (Barreiro et al. 2006, Navarro-Antolin et al. 2007, MacMillan-Crow et al. 1999).

It is known that MnSOD eliminates ROS more effectively in intact mitochondria than in those that are damaged (Raha et al. 2000). We therefore analyzed additional mitochondrial marker, PDH in mitochondrial and cytosolic fractions to confirm the integrity of isolated mitochondria and their vulnerability to disruption during the isolation procedure. Low levels of MnSOD and PDH in the cytosolic fraction from all experimental groups indicate good preservation of mitochondria during their isolation in this study. Neither CIH nor NAC treatment affected the sensitivity of the mitochondrial inner membrane to disruption.

In conclusion, the up-regulation and activation of mitochondrial MnSOD in close correlation with the reduction of myocardial infarct size suggest that this enzyme can contribute to the mechanism of CIH-induced tolerance against ischemia/reperfusion injury. Blunting of these effects by treatment with NAC supports the view that ROS-dependent signaling during hypoxic adaptation plays an important role in this form of cardioprotection.

Conflict of Interest
There is no conflict of interest.

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