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Enhancement of Superoxide Dismutase Activity in Rat Lungs after Hypoxic Preconditioning

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Abstract

Ischemic preconditioning has been proved to reduce tissue damages and benefit subsequent organ transplantation. Chronic hypoxic preconditioning was found to increase the levels of lung antioxidants. This study was to test the hypothesis that levels of lung antioxidants might increase after hypoxia which may counteract the insults of free radicals. Female Wistar rats were kept in an altitude chamber (380 torr) 15 h a day for 4 weeks (hypoxia-adapted). Controls were kept at room air pressure (sea-level). After hypoxic preconditioning, no significant difference in the levels of the oxidative markers, malondialdehyde, thiobarbituric acid reactive substances and isoprostane was seen in the lungs of the hypoxia-adapted rats compared to the sea-level controls. Both the activity and protein level of manganese superoxide dismutase were higher in hypoxia-adapted lungs. Lung manganese superoxide dismutase mRNA levels, determined by real-time RT-PCR, were not significantly different in the two groups of rats. When isolated saline-perfused lungs were prepared and treated with xanthine (500 μM) and xanthine oxidase (5 mU/ml), and the levels of free radicals in the perfusate determined by chemiluminescence, less chemiluminescence was seen in the hypoxia-adapted lung perfusate. When the vascular response was determined in this same preparation before or 45 min after xanthine/xanthine oxidase treatment, the filtration coefficient was increased in the sea-level lungs but not in the hypoxia-adapted lungs. We conclude that an increase in superoxide dismutase activity and protein levels is one of the benefits of hypoxic preconditioning.

Key Words: hypoxic preconditioning, isolated lung, superoxide dismutase, xanthine oxidase

Introduction

Ischemic preconditioning could confer protection against lung damage associated with organ transplantation (3, 30). More recently, studies have suggested that hypoxia can substitute for ischemia as preconditioning (4, 33). Ischemic preconditioning is a phenomenon by which multiple brief exposures to ischemia can reduce the damage caused by subsequent prolonged ischemia (19). Rats exposed to 10-11% oxygen for 7 days develop tolerance to hyperoxia and can survive for a prolonged period in 100% oxygen, and this preexposure to hypoxia is associated with increased lung manganese superoxide dismutase (MnSOD) activity, but not in the activities of CuZnSOD, glucose-6-phosphate dehydrogenase, cytochrome oxidase or succinate cytochrome C reductase (12). The benefit of hypoxic preconditioning seems to be the result of enhanced SOD expression, but this is not always the case. After 7 days of hypoxia, lung MnSOD activity decreases significantly in male, but not female, mice (29). Another study has shown that 7 days of hypoxia has a negligible effect on SOD activity in both pregnant and non-pregnant rats (24). A recent study showed that acute hypoxia/reoxygenation induced increases in the expressions

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of SOD and catalase in lung tissues (26).

Free radical attacks lipids, proteins, sugars and nucleic acids leading to the formation of bioproducts the detection of which in fluids and tissues represents the currently available method for assessing oxidative damage. Detection of lipid oxidation is more traditional and simple (9). Hypoxia has been shown to generate oxygen radicals which may be involved in the structural remodeling of peripheral pulmonary arteries and pulmonary hypertension (1, 10, 21, 31). Hoshikawa *et al*. (8) found that lung xanthine oxidase (XO) activity in rats was elevated throughout the entire course of hypoxia and inhibition of XO significantly reduced hypoxia-induced right ventricular hypertrophy and pulmonary vascular thickening; they also reported that levels of the oxidative marker, phosphatidylcholine hydroperoxide, were only increased during the first week of hypoxic exposure. The aim of the present study was to test the hypothesis that levels of lung antioxidants might increase to counteract the increase in oxidants observed during the later stage of chronically intermittent hypoxia.

Materials and Methods

Induction of Chronic Hypoxia

Female Wistar rats weighing 180-200 g were used. Animal care and the experimental protocol were in accordance with the guidelines of the National Science Council of the Republic of China (NSC 1997). The rats were kept in an altitude chamber for 15 h a day for 4 weeks at a constant temperature and on a light cycle (light at 07:00 to 18:00) as previously described (4). Controls were kept at room air pressure (sea level, SL) while the hypoxia-adapted (HA) rats were exposed to 380 torr from 17.00 to 8.00 h, then returned to room air; this pressure represents an altitude of 5,500 m, the maximal altitude at which most rats can adapt successfully. All animals were allowed free access to food and water at all time and their body weight was measured weekly.

Lung Treatment for Biochemical Tests

After this period of exposure, the animals were anesthetized with sodium pentobarbital (40 mg/kg) and the lungs were removed and treated differently for the various measurements. In general, the lung tissue was homogenized on ice in 4 ml of Tris-EDTA buffer using a Polytron. Plasma membrane preparations were prepared as previously described (18). Total protein in the membrane preparation was quantified using the Bio-Rad Protein Assay according to the manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA, USA) using BSA as a standard.

Malondialdehyde (MDA) Assay

MDA levels in the lung were measured spectrophotometrically according to the method provided with the kit from Oxis Research (Portland, OR, USA), and the MDA levels were expressed as mM per mg of protein.

Thiobarbituric Acid Reactive Substances (TBARS) Assay

TBARS levels in the lung were measured spectrophotometrically at 532 nm using a commercial kit from Zeptometrix (Buffalo, NY, USA) and were expressed as mM per mg of protein.

Isoprostane Assay

The extraction and enzyme immunoassay procedures used to measure isoprostanes were those recommended in the 8-iso-prostaglandin $F_{2\alpha}$ enzyme immunoassay kit provided by Assay Designs Inc. (Ann Arbor, MI, USA). HCl (2 M) was added to all samples (pH 3.5) which were then mixed and centrifuged applied to a Sep-Pak C18 column with washes of water and hexane, followed by elution of the isoprostanes which was then dried off under nitrogen and the samples reconstituted in 1 ml of assay buffer. For the assay, standards and samples were added in duplicates to the 96-well plate provided in the kit, followed by sequential addition of tracer and antibody and incubation for 45 min at room temperature. After several washes with the wash buffer and optimal color development, the optical density of the wells was read at 450 nm; the values for the samples were expressed as picograms per mg of protein.

Lung SOD Activity

SOD activity was determined using a BIOXYTECH® SOD-525™ kit (OXIS International, Portland, OR, USA). This method is based on the SOD-mediated increase in the rate of autoxidation of 5,6,6a,11b-tetrahydro-3,9,10-trihydrobenzo[c] fluorene (R1) in an aqueous alkaline solution to yield a chromophore with a maximal absorbance at 525 nm (A_{525}) (9). The lungs were first perfused with 0.9% NaCl containing 0.16 mg/ml of heparin to remove red blood cells and were then homogenized and centrifuged as described above. Part of the supernatant was reacted with an extraction reagent (ethanol/chloroform 62.5/37.5, v/v; 25 volumes of supernatant: 40 volumes of reagent) to inactivate the manganese (Mn) SOD and iron (Fe) SOD in order to measure copper (Cu) and Zinc (Zn) SOD activity. SOD activity was determined from the ratio of the autoxidation rates in the presence and in the absence of SOD. Two separate

samples per lung were assayed, one for total SOD activity and the other for Cu/ZnSOD activity; MnSOD activity was calculated as the difference between the two.

Immunoblotting for Cu/ZnSOD, MnSOD in the Lungs

Cytosolic proteins were prepared from the lung and from the liver that was used as a positive control. All procedures were performed at 4°C or on ice. The tissues were rinsed in saline and homogenized in 50 mM Tris, pH 7.4, containing protease inhibitors (10 mM PMSF, 10 μM benzamidine, 10 μM leupeptin, and 1 μg/ml of trypsin inhibitor, Sigma, Saint Louis, MO, USA). The homogenate was then centrifuged at 12,000 g for 20 min or at 1,000 g for 5 min to prepare supernatants for Cu/ZnSOD or MnSOD immunoblotting, respectively; the supernatants were stored at -70°C until analyzed. A sample of the supernatant (40 μg protein) from each preparation was applied to SDS polyacrylamide gels under denaturing conditions, and the separated proteins were electrophoretically transferred to a nitrocellulose membrane (Amersham, Buckingham, England, UK). The membranes were then incubated overnight at 4°C with a 1/1,000 dilution of polyclonal antibodies for MnSOD or Cu/ZnSOD (The Binding Site, Birmingham, UK), washed, and then incubated for one hour at room temperature with biotinylated donkey anti-sheep/goat IgG antibodies conjugated to horseradish peroxidase (Vector, Burlingame, CA, USA). After washes, the bound antibody was visualized using a commercial 3, 3'-diaminobenzidine substrate kit (Vector, Burlingame, CA, USA) and the density of the labeled band in each lane was determined semi-quantitatively by densitometry using an image analyzing system (Alpha Innotech, San Leandro, CA, USA). The data in each group are presented as the means ± standard error for the integrated digital value (IDV) × area^{-1} of each lane.

Quantitative Real-Time Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) to Measure MnSOD mRNA Levels

Total RNA from the lung of six SL rats and five HA rats was extracted using the TRIZOL reagent (Gibcobrl, Grand Island, NY, USA) following the manufacturer's instructions. The amount of total RNA was determined from the 260/280 optical density ratio of each sample, and its quality confirmed by electrophoresis on 1.2% agarose gels stained with ethidium bromide. The PCR procedure was performed using a TaqMan EZ RT-PCR kit (Perkin-Elmer, Foster City, CA, USA) as previously described (17).

The theoretical basis and methodology of the ABI PRISM 7700 Sequence Detection System (TaqMan) real-time quantitative PCR (Perkin-Elmer Applied Biosystem, Foster City, CA, USA) have been described by Johnson *et al*. (11). Briefly, samples with a high starting copy number of the gene of interest show increased fluorescence early in the PCR process resulting in a low threshold cycle (C_T) number. The comparative C_T ($\Delta\Delta C_T$) method was used to quantify lung MnSOD mRNA levels, the fold-induction being equal to 2-($\Delta\Delta C_T$).

The primers and fluorogenic probes for MnSOD and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were designed and total RNA extraction and the PCR procedure were performed as previously described (17). The MnSOD primers and probe were: forward primer, 5'-ACG CGA CCT ACG TGA ACA ATC T-3'; reverse primer, 5'-CAG TGC AGG CTG AAG AGC AA-3'; and the probe, 5'-6-carboxy-flurescein (FAM)-ATC TCC CTT GGC CAG GGC CT-6-carboxy-tetramethylrhodamine (TAMRA)-3'. The GAPDH primers and probe were: forward primer, 5'-TTT CTC GTG GTT CAC ACC CA-3'; reverse primer, 5'-GTC ATC ATC TCC GCC CCT T-3'; and the probe, 5'-FAM-CGC TGA TGC CCC CAT GTT TGT G-TAMRA-3'.

Isolated Lung Setup

In some animals, isolated-perfused lungs were prepared as previously described (14). Briefly, after insertion of a tracheal cannula, the chest was opened and the lungs were ventilated with a humidified 95% air and 5% CO_2 gas mixture under a respiratory rate of 60 times/min, a tidal volume of 2 ml and a final expiratory pressure of 2.5 cm water. After injection of the right ventricle with heparin (150 IU), the pulmonary artery was cannulated and perfused with Krebs-Henseleit buffer (118 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2 \cdot H_2O$, 1.2 mM $MgSO_4 \cdot 7H_2O$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, and 10 mM glucose) containing 4 g/100 ml of bovine serum albumin. A wide-bore cannula was placed in the left atrium through the left ventricle to collect the effluent perfusate for recirculation. A perfusion rate of 3 ml/min/100 g body weight was maintained by a roller pump through an air bubble trap. The heart and the lungs were then removed *en bloc* and placed on a weighing pan which was mounted on a Grass force transducer, which was suspended in a constant-temperature (37°C) humidified chamber, to detect changes in the weight of the lung. The weighing system was calibrated by placing a 2-g weight on the pan and adjusting the output to 5 cm of chart deflection. The pulmonary arterial (P_{pa}) and venous (P_v) pressures were monitored continuously with Statham pressure transducers which were placed at the same height as the heart. The

distance between the pressure transducers and the pulmonary artery or vein was 29 or 50 cm, respectively. The resistances of the connecting catheters were measured and the values for the P_{pa} and P_v described above were corrected for these resistances. Changes in the weight of the lung, P_{pa} and P_v were continuously recorded using a Grass recorder.

Capillary pressure (P_c). Using constant-flow perfusion, venous outflow was momentarily stopped for 3-4 sec at end expiration resulting in a rapid rise in P_v, followed by a slower, but steady, rise. The value for the P_c was obtained by extrapolating the slow rising component back to zero time.

Filtration coefficient (K_{fc}). The K_{fc} was determined by the gravimetric method of Drake *et al.*. (6) Once an isogravimetric state was achieved, the P_v was rapidly increased by 10 cmH_2O for 10 min. This hydrostatic pressure caused a rapid, followed by a slow but steady, rise in lung weight. The rapid component represented the expansion of pulmonary blood vessels whereas the slow component was due to fluid filtration into the interstitial space. The initial rate of fluid filtration was estimated by extrapolating the slow component to zero time on a semilog plot. The value of the y intercept was then divided by the hydrostatic pressure challenge (ΔP_c) and normalized to 100 g of lung weight.

Experimental protocols. We studied the pulmonary vascular response to exposure to xanthine/xanthine oxidase (X/XO). At the end of a 20-min equilibration (baseline) period, the baseline values for the vascular parameters (P_{pa}, P_v, P_c, and K_{fc}) were measured, then xanthine (500 μM) was added to the perfusate followed 5 min later by the addition of XO (5 mU/ml). The vascular response was determined before and 45 min after treatment and the arterial resistance [R_a, $R_a = (P_a - P_c)$/perfusion rate], venous resistance [R_v, $R_v = (P_c - P_v)$/perfusion rate] and K_{fc} were calculated.

Chemiluminescence Assay

In order to confirm the release of free radicals after X/XO treatment, free radicals were determined using a Chemiluminescence (CL) Analyzing System (CLD-110, Tohoku Electronic Industrial Co., Sendai, Japan) as described before (17). 0.2 ml samples obtained from the buffer and perfusate 5 min and 45 min after addition of X/XO were immediately wrapped with aluminum foils and kept in the iced box until the CL measurement which was usually done within 2 h. Five groups of samples were prepared: [1] Buffer-X/XO, with addition of X/XO to the buffer; [2] Buffer-SOD-X/XO, with addition of 10,000 U of SOD before application of X/XO; this group was intended for confirmation of the enhanced CL that was originated from superoxide; [3] Time control; [4] SL-X/XO, with addition of X/XO to the perfusate of SL lungs; [5] HA-X/XO, with addition of X/XO to the perfusate of HA lungs.

CL was measured in the completely dark chamber of the Chemiluminescence Analyzing System. After 100 s, 1.0 ml of 0.1 mM lucigenin (bis-N-methylacridinium nitrate, Sigma) in PBS (pH 7.4) was injected into the cell and the CL of the sample was continuously measured for 600 s. The total amount of CL over this period was calculated by integrating the area under the curve and subtracting the background level, equivalent to the dark average. The assay was performed in duplicates for each sample and the results were expressed as CL counts/10 s. The means ± SE (standard error) for the CL counts for all the samples in each group was calculated.

Statistical Analysis

Values are expressed as the means ± SE. Differences in parameters between groups were analyzed

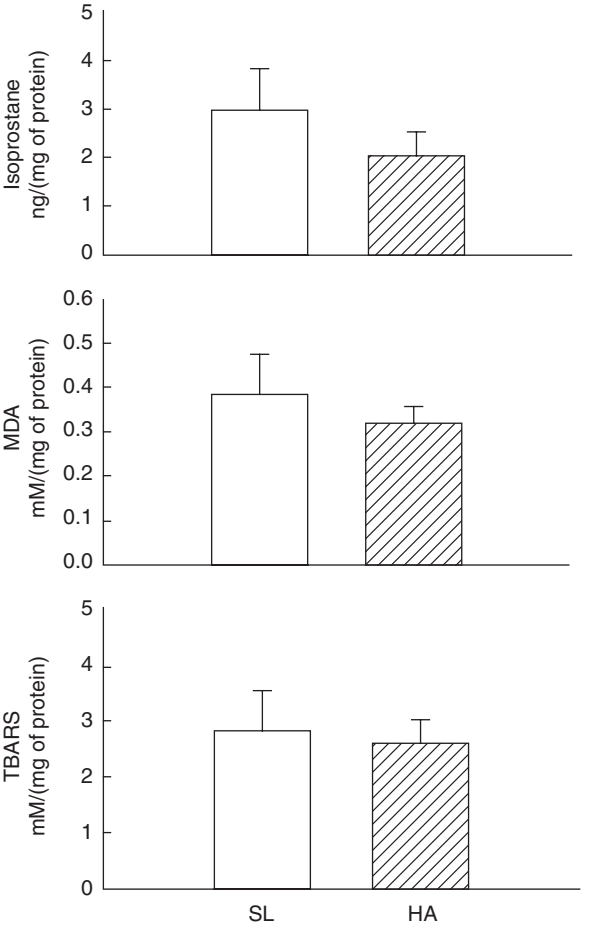

Fig. 1. Lipid peroxides in the lungs of sea level (SL, n = 6) and hypoxia-adapted (HA, n=6) rats. MDA, malondialdehyde; TBARS, thiobarbituric acid reactive substances.

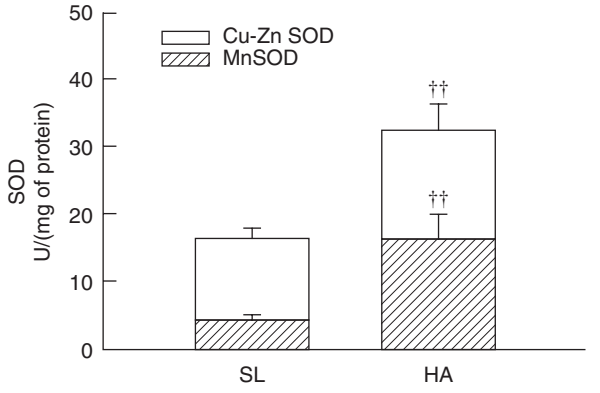

Fig. 2. Superoxide dismutase (SOD) activity in the lungs of sea level (SL, n = 10) and hypoxia-adapted (HA, n = 10) rats. ††: $P < 0.01$ comparing the SL and HA lungs.

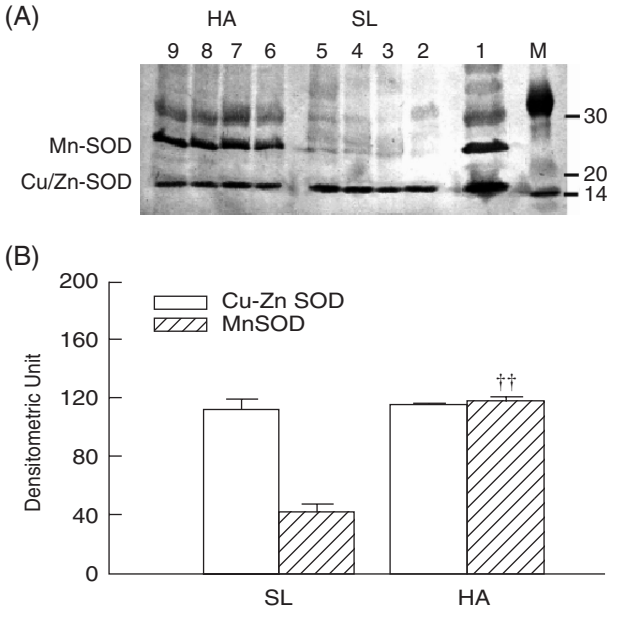

Fig. 3. Superoxide dismutase (SOD) protein levels in the lungs from sea level (SL) and hypoxia-adapted (HA) rats. (A) Western blot of the plasma membrane fraction prepared from 4 SL lungs (lanes 2-5) and 4 HA lungs (lanes 6-9). Lane M, prestained molecular weight standards. Lane 1 is the positive control from the liver. (B) Semi-quantitative densitometry of the above results. ††: $P < 0.01$ comparing the SL and HA lungs.

using the non-paired Student's *t*-test. Differences between values before and after X/XO challenge were analyzed using the paired Student's *t*-test. Differences were considered significant when the *P* value was < 0.05.

Results

After 4 weeks of chronic hypoxia, the hematocrit increased to 60.2 ± 1.6% compared to 45.2 ± 1.4% in

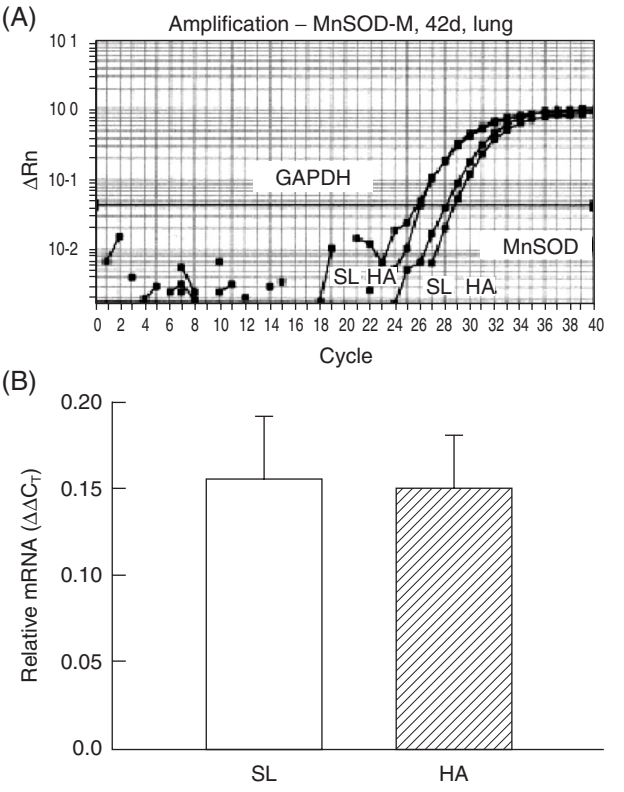

Fig. 4. Superoxide dismutase (SOD) mRNA expression in lungs from sea level (SL) and hypoxia-adapted (HA) rats measured using real-time RT-PCR. (A) A typical amplification plot for SOD and glyceraldehyde-3-phosphate dehydrogenase (GADPH) mRNAs. The four amplification plots show RNA samples from one SL and one HA lung amplified using specific GAPDH and NK-1 receptor primers. The graph shows the fluorescence intensity above the baseline (ΔRn) for each PCR plotted against cycle number. (B) Fold-induction in 5 SL and 5 HA lungs. The $\Delta\Delta C_T$ value was calculated by subtracting the ΔC_T value for the no-template control from that for each sample.

SL rats while the body-weight gain was less (12 ± 5 g compared to 45 ± 7 g in SL rats).

No significant changes were seen in isoprostane, MDA or TBARS levels in the lungs after 4 weeks of hypoxia (Fig. 1).

SOD activity in the lungs is shown in Fig. 2. Total SOD activity was higher after chronic hypoxia, this increase being due to a 4-fold increase in MnSOD activity in HA rats compared to the SL controls. The Cu/ZnSOD activity was not significantly different between the 2 groups.

Western blot analysis of the SOD protein levels also revealed higher protein levels of MnSOD in HA lungs with no changes in Cu/ZnSOD levels (Fig. 3).

Fig. 4A shows the original amplification traces for the C_T values for MnSOD and GAPDH mRNAs, while Fig. 4B shows that there was no significant

Table 1. Release of chemiluminescence (CL) into the perfusate of isolated lungs from sea level-adapted (SL) and hypoxia-adapted (HA) rats treated with xanthine/xanthine oxidase (X/XO)

Parameters / Groups	Baseline (CL/10 sec)	X/XO-5 min (CL/10 sec)	X/XO-45 min (CL/10 sec)
SL-Time control (n = 3)	574.4 ± 282.8	275.4 ± 166.2	292.1 ± 135.3
Buffer-X/XO (n = 6)	157.4 ± 31.9	7,737.8 ± 960.3*#	2,494.1 ± 257.8*#
SL-X/XO (n = 7)	733.7 ± 213.7	3,778.9 ± 425.4*	1,582.7 ± 193.3*
HA-X/XO (n = 7)	276.8 ± 128.1	1,852.0 ± 270.4*#	990.5 ± 214.1*

Buffer-X/XO: X/XO added to perfusate. X/XO-5 min and X/XO-45 min: 5 min and 45 min after treatment with X/XO. *$P < 0.05$ compared to baseline value using Student's paired *t* test; #$P < 0.05$ compared with SL-X/XO value.

Table 2. Effects of xanthine/xanthine oxidase (X/XO) on the pulmonary hemodynamics in isolated lungs from sea-level (SL) and hypoxia-adapted (HA) rats

Parameters / Groups	P_{pa} (mmHg)		P_v (mmHg)		P_c (mmHg)		K_{fc} (g/min·mmHg·100 g)		R_a (mmHg/ml·min)		R_v (mmHg/ml·min)	
	Baseline	X/XO	Baseline	X/XO	Baseline	X/XO	Baseline	X/XO	Baseline	X/XO	Basline	X/XO
SL (n = 7)	10.6 ± 0.41	11.14 ± 0.35*	0.05 ± 0.05	0.33 ± 0.04	3.33 ± 0.27	3.10 ± 0.15	0.34 ± 0.06	0.9 ± 0.10*	0.97 ± 0.03	1.07 ± 0.03*	0.38 ± 0.02	0.38 ± 0.01
HA (n = 8)	11.30 ± 0.37	11.97 ± 0.36*	0.7 ± 0.12	0.45 ± 0.09	3.85 ± 0.30	3.37 ± 0.20	0.35 ± 0.06	0.43 ± 0.09	1.15 ± 0.03	1.29 ± 0.06*	0.47 ± 0.04	0.46 ± 0.02

P_{pa}: pulmonary arterial pressure, P_v: pulmonary venous pressure, P_c: pulmonary capillary pressure, R_a: pulmonary arterial resistance, R_v: pulmonary venous resistance, K_{fc}: pulmonary filtration coefficient, n: number of lungs. *$P < 0.05$, compared with baseline.

difference between the two groups in terms of the fold-induction values for the lung mRNA.

We used CL to measure the free radical released in the samples (Table 1). X/XO increased the CL counts significantly even 45 min after the treatment; this increase was largely reduced with prior addition of SOD. The baseline CL count in the HA lung perfusate was lower than, but not significant different from, that in the SL lung perfusate. Five min after X/XO administration, the increase in the CL count in the HA lung sample was only half that in the SL lung perfusate ($P < 0.05$) and only a quarter of that in the buffer. Forty-five min after X/XO treatment, the CL count was reduced in all samples with no significant difference between the SL and HA lung perfusate, but a significantly higher CL count in the buffer sample was observed.

As shown in Table 2, in both groups of lungs, X/XO treatment resulted in a significant increase in both P_{pa} and R_a with no significant changes in P_v, R_v or P_c. It is interesting to note that X/XO treatment resulted in a significant increase in the K_{fc} in the SL lungs, but not in the HA lungs.

Discussion

Prolonged exposure to high altitude is reported to cause an increase in expired pentane; this is considered to be due to an increase in lipid peroxidation which can be reversed by administration of vitamin E (28). MDA levels in rat lungs do not change until day 21 of hypoxic exposure (20). However, Hoshikawa *et al.* (8) reported that lung phosphatidylcholine hydroperoxide in rats was increased in the early stage of hypoxia (days 1 and 7), but not on days 14 and 21. Whether chronic hypoxia leads to changes in oxidative stress is controversial. We found that the levels of the oxidative markers, MDA, TBARS and isoprostane were not significantly changed. These results suggest that following exposure to long term (4 weeks) hypoxia, the same extent of oxidative injury is seen in the lungs of chronic HA or SL control rats.

Free radicals may be derived from a variety of sources. Oxidative damage in a system is determined by the balance between free radicals and antioxidants. Whether chronic hypoxia releases more free radicals is rarely discussed. XO uses molecular oxygen as an electron acceptor and generates superoxide during the oxidation of hypoxanthine (7). Hoshikawa *et al.* (8) reported that during chronic hypoxia, lung XO activity was increased from day 1 to day 21, especially during the initial 3 days of the hypoxic exposure. If the increase of xanthine oxidase also occurred in the HA lungs in our study, however, the oxidative injury did not increase on the 4 weeks of hypoxia. Thus, we expected an increase in the antioxidants.

No significant difference was seen in Cu/ZnSOD activity and protein in the SL and HA lungs, but an increase in both the activity and protein levels of the lung MnSOD was seen in the HA lungs, a result similar to that of Sjostrom and Crapo (29). Both results differ from those of other reports (20). The

reason for this discrepancy is not clear but may be due to the animals used, the duration of hypoxia, or the chemical measurements methods. One surprising finding in the present study is that the significant increase in MnSOD activity and protein level seen in the HA lungs was not accompanied by an appreciable change in mRNA levels suggesting that post-transcriptional regulation plays an important role in antioxidant adaptation. Other studies in mice (24) or rabbits (25) have also found that the change in the lung MnSOD level after hypoxia is not necessarily related to its mRNA.

In this *in vitro* study, using lucigenin-enhanced CL counts would be a reliable assay for free radicals. We previously demonstrated that using lucigenin or 2-Methyl-6-[4-methoxyphenyl]-3,7-dihydroimidazo-[1,2-a]-pyrazin-3-one hydrochloride (MCLA) to enhance the CL counts would produce similar results in *in vivo* (5). The baseline CL detected in the perfusate was lower in the HA lungs but the difference was not significant. After X/XO treatment, HA lungs released less CL showing that the free radicals were scavenged by higher levels of lung antioxidants. Another concern is whether all the CL detected in the perfusate was due to direct production by the injected X/XO. If X/XO stimulated lung tissue to release more reactive oxygen species, the HA lungs would then be less responsive.

The lungs with X/XO treatment resulted in only a slight increase in the P_{pa} with no changes in the P_c. In most previous studies (13, 15), a very large dose of XO was used which induced severe pulmonary vasoconstriction and increased capillary pressure. These studies quantified injury by measuring gain in the weight of the lung which might be independent of injury to the pulmonary microvascular membrane. In the present study, instillation of small doses of XO into the perfusate increased the pulmonary microvascular endothelial permeability in the SL control lungs, but not in HA lungs, as measured by the capillary filtration coefficient which is a more sensitive index of endothelial permeability that is independent of changes in vascular pressures. We found that the X/XO-induced increase in the value of the pulmonary filtration coefficient was attenuated in the HA lungs.

X/XO reduces O_2 to superoxide and subsequently to hydrogen peroxide. Although hydrogen peroxide has been proposed as the main cytotoxic substance responsible for lung injury (13, 32), superoxide may react directly with cellular targets or combine with nitric oxide to form peroxynitrite, an agent known to cause lipid peroxidation and sulfhydryl oxidation (23), thus, producing damages.

The lungs in HA rats suffered less injury on exposure to X/XO because of an increase in the levels of radical scavengers. Since the isolated lungs had their vasculature washed free of blood, the protective effect of hypoxia is probably due to an effect on resident lung cells rather than on circulating cellular or humoral elements.

In addition to SOD, other antioxidants may play some roles in the beneficial effect of hypoxic preconditioning. Pre-exposure to hypoxia increases survival in rats subsequently exposed to continuous hyperoxia (29, 32). In addition, lungs isolated from hypoxia-exposed rats develop less acute edematous injury when perfused with hydrogen peroxide because of increased glutathione redox cycle activities (6, 27). This is similar to our own findings in the brain which showed that although the basal GSH content, GSH/GSSG ratio and SOD and catalase activities were not altered by hypoxia, hypoxic treatment not only reversed the iron-induced reduction in the GSH content, GSH/GSSG ratio and SOD activity but also inhibited the iron-induced increase in catalase and glutathione peroxidase activities found in normoxic rats (16).

Hypoxia preconditioning may have other beneficial effects (2, 22) that protect the lungs from detrimental challenge, *e.g.* heat-shock protein and Bcl2 are over-expressed in hypoxic kidneys (4), thus, reducing renal apoptosis during ischemia/reperfusion.

We conclude that 4 weeks of intermittent hypoxia preconditioning did not increase the levels of oxidative damage due to an increase in the MnSOD activity and protein level. This increase was not due to an increase in its mRNA levels.

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