HK J Paediatr (new series) 2000;**5**:3-7

Original Articles

Antioxidant Enzyme Activities and Antioxidant Enzyme Gene Expression in Hyperoxia-induced Lung Injury in Premature Rat

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Abstract In a preterm rat model of hyperoxia-induced lung injury, we studied the lung antioxidant enzyme (AOE) activities and gene expression. After seven days of continuous exposure to >95% oxygen, severe injuries were found in the lungs with inflammation and edema. Manganese superoxide dismutase (MnSOD) mRNA level and SOD activities in lung tissues were significantly elevated when compared to the room air controls ($p=0.002$, $p<0.001$). There was no significant increase in the levels of glutathione peroxidase (GP), catalase (CAT) or their gene expression. These findings show that in the premature rat lung, oxygen-induced damage is associated with upregulation of SOD gene expression and increased SOD activities.

Key words Premature lung; Antioxidant enzymes; Gene expression; Hyperoxia

Introduction

Preterm infants exposed to high concentration oxygen are prone to develop hyperoxic lung damage, which is an important underlying cause of bronchopulmonary dysplasia (BPD).[1] Although the causative agent for BPD has not been conclusively identified, hyperoxia-induced lung injury is believed to be a major factor. Data from both cellular and whole animal models suggest that hyperoxic lung damage produces pathological changes similar to those seen in BPD.

Among the principal cellular defenses against hyperoxic lung damage is the antioxidant enzyme (AOE) system which includes the manganese superoxide dismutase (MnSOD), copper-zinc superoxide dismutase (CuZnSOD), catalase (CAT), and glutathione peroxidase (GP).[2] The SOD, CAT, GP offer protection against intracellular oxidative stress. The enzymes are scanty in the lungs of preterm fetuses and become more abundant as gestation increases. Deficiency in these enzymes may play a primary role in the pathogenesis of oxygen-induced lung injury and BPD is commonly seen in the extremely preterm infants. Previous studies have shown that enhancement of pulmonary AOE such as SOD, CAT and GP has a protective effect on the lung of adult or full-term animal against oxygen toxicity.[3,4] There is however little information on the change of AOE activities and AOE gene expression in premature neonate under hyperoxic condition.[5,6] By using a premature rat model, we studied the AOE activities and AOE gene expression under hyperoxia and normative condition.

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Received August 5, 1999

Materials and Methods

Hyperoxia Exposure Experiments

The study was approved by the Committee on Research Animal Welfare of the Chinese University of Hong Kong. Preterm rats were delivered by hysterotomy at 21 days of gestation. The procedures and condition of hyperoxia exposure experiment were as previously described.[7] Briefly, in each experiment, premature pups were randomly divided into two groups each consisting of 12 pups. Each group was housed in a plastic exposure chamber, which was at all times filled with either >95% oxygen (hyperoxia group) or room air (room air group). The interior of the exposure chamber was continuously monitored for its oxygen (O_2 >95%, Miniox 3 Oxygen

Monitor, Mine Safety Appliances Co, USA), carbon dioxide concentration (CO_2<0.5%, Fyrite Gas Analyser, Bacharach Inc, USA), temperature (25±1°C), and relative humidity (50%-80%). The animals were sacrificed on day seven of life. The lungs were dissected or perfused with ice-cold 0.9% saline. The perfused lung was then blotted dry, and quickly frozen in liquid nitrogen until analysis. The right lungs were used for the assay of AOE activities, and the left lungs for the assay of AOE mRNA.

Analysis of AOE Activities

Seven lung samples in each group were homogenized in freshly prepared cold buffer (2.5 mM potassium phosphate, 0.1 mM EDTA, pH 7.8). After removal of a 500 µl sample from the lung homogenates for DNA estimation, the remaining solution was centrifuged at 75000 g for 1 hour at 4°C (Tabletop Ultracentrifuge, Beckman instrument Inc, USA). The supernatant was assayed for activities of total lung SOD using the method of inhibition of nitrite formation from hydroxylammonium chloride.[8] CAT using the method of H_2O_2 consumption rate.[9] GP activity was measured using a modified method of Hafeman.[10] The enzyme activities were estimated with a DU650 spectrophotometer (Beckman instrument Inc, USA), and expressed as activity unit per mg DNA. Commercially available purified SOD, CAT and GP enzyme standards (Sigma Chemical Co, St. Louis, USA) were used for calibration. DNA was measured by reading the absorbance value at 260 nm using GeneQuant RNA/DNA calculator (Pharmacia LKB Biochrom Ltd, England).

Analysis of AOE Gene Expression

Total RNA was extracted from the isolated lung tissue of four randomly selected animals in each group by the acid guanidinium-phenol-chloroform technique.[11] Oligonucleotide primer and probe sequences of rat MnSOD, CuZnSOD, CAT, GP and β-actin were designed from the published sequences[12-16] and synthesized by Gibco BRL, USA (Table 1). We used the β-actin gene as an internal control to standardize the reverse-transcription polymerase chain reaction (RT-PCR) products. One microgram of total RNA was used for the synthesis of 20 µl first-strand cDNA by reverse transcriptase (Gibco BRL, USA) and oligo-P (dT)15 (Boehringer Mannheim, Germany). The 50 µl PCR reaction mixture contained 10 µl diluted cDNA, the upstream and downstream primers (150 nM), dNTPs (100 uM), and 1.25 units of Thermus aquatus DNA Polymerase (Gioco BRL, USA). A thermal cycler (The PTC-100, MJ Research, Inc, USA) performed the amplification for 29 cycles with denaturation at 94°C for 1 min, annealing at 58 °C for 45 sec, and extension at 72 °C for 45 sec. The PCR products were separated by electrophoresis. Single band corresponding to the predicted size of the amplified products were identified under an ultraviolet transilluminator. The amplified products were transferred to a nylon filter membrane, and hybridized with ECL-labeled probe (3'-oligolabelling and detection systems Kit, Amersham UK). The probes hybridized only to the bands, which corresponded in size to the ethidum bromide-stained gels. The band densities were scanned with a densitometer (model GS-670 imaging

Table 1 Sequences of primers and probes

		Oligonucleotide sequences	Predicted cDNA size
PCR primer			
MnSOD	upstream	5'-GTGGTGGAGAACCCAAAGGA-3'	238bp
	downstream	5'-GCGTGCTCCCACACATCAAT-3'	
CuZnSOD	upstream	5'-ATGGGGACAATACACAAGGC-3'	225bp
	downstream	5'-TCATCTTGTTTCTCGTGGAC-3'	
CAT	upstream	5'-GTCCGATTCTCCACAGTCGC-3'	272bp
	downstream	5'-CGCTGAACAAGAAAGTAACCTG-3'	
GP	upstream	5'-CACAGTCCACCGTGTATGCC-3'	292bp
	downstream	5'-AAGTTGGGCTCGAACCCACC-3'	
β-Actin	upstream	5'-TCACTATCGGCAATGTGCGG-3'	260 bp
	downstream	5'-GCTCAGGAGGAGCAATGATG-3'	
Probes for hybridization			
MnSOD		5'-CCTTGCAGTGGGTCCTGATT-3'	
CuZnSOD		5'-CGGCCAATGATGGAATGCTC-3'	
CAT		5'-TCCCACAAGGTCCCAGTTAC-3'	
GP		5'-GGTCGGACATACTTGAGGGA-3'	
β-actin		5'-TCAGCGATGCCTGGGTACAT-3'	

densitometer, Bio-rad instrument Ltd, USA). The relative amount of mRNA in each sample was calculated from the densitometry ratio of samples OD value/β-actin OD value. The technique for PCR used in this study was carefully chosen to ensure that the amount of product synthesized was proportionally related to the amount of mRNA in the original preparation.[17]

Lung Microscopic and Ultrastructural Studies

The lungs of 10 randomly selected animals in each group were dissected for microscopical and ultrastructural examination. Histological specimens were fixed in 10% buffered formalin, embedded in paraffin, stained with standard hematoxyline-eosin, and examined under the light microscope. For ultrastructural examination by electron microscope, samples were fixed in 2.5% glutaldehyde fixative, postfixed with 2% osmium tetroxide, and then dehydrated in a graded series of ethanol. The samples were then embedded and stained with lead citrate and uranyl acetate. Each section was examined under a Philips CM 100 electron microscope (Philips Ltd, Holland).

Statistical Analysis

Quantitative comparison of parameters in the two groups was carried out using non-paired Student' t test. Differences were considered significant if $p<0.05$. All values are express as mean±SD.

Results

Table 2 shows the pulmonary AOE activities in the premature rats exposed to >95% oxygen or room air for seven days. SOD activity was significantly greater in the hyperoxia group ($p<0.001$). The activities of GP and CAT also showed an increasing trend in favour of the hyperoxia group, although the differences between the two groups did not reach statistical significance ($p=0.168$ and $p=0.481$, respectively).

The results of the semi-quantitative southern blot analysis for mRNA for each of the four AOE is shown in Figure 1. Compared to the room air group, MnSOD mRNA expression was significantly higher in the hyperoxia group ($p=0.002$). There was no significant difference in CuZnSOD, CAT, or GP mRNA expression between the two groups ($p=0.362$, $p=0.347$ and $p=0.126$, respectively), although the mean CuZnSOD and GP mRNA expression showed an increased trend in favour of the hyperoxia group.

Histological examination by light microscope revealed the presence of interstitial edema, hemorrhage, and diffused alveolar damage in the lungs of all animals in the hyperoxia group. Ultrastructural examination demonstrated the presence of damage in pneumocytes as well as interstitial edema (Figure 2). In contrast both light and electron microscopy findings of the lungs in the room air group were normal.

Discussion

In this study, we used a preterm rat model to study the effect of hyperoxia on the change of lung AOE activities and the AOE gene expression. After seven days of exposure to >95% oxygen, the lungs of the preterm pups had sustained significant lung damage as evidenced by light microscopical and ultrastructural changes. These results are consistent with the findings of previous reports on adult and full-term animals.[4]

The premature rat provides a convenient model for this study of the mechanism of the effects of hyperoxia on the pulmonary AOE system on the molecular level, which has remained incompletely understood. Our results demonstrated that after exposure to >95% oxygen for seven days, the MnSOD mRNA and SOD activities increased significantly when compared to those in the room air controls. This indicates that during hyperoxia stress, pertranslation control may participate in regulating the SOD gene expression in premature rat lungs exposed to hyperoxia. It is unclear whether the increased level of pulmonary SOD mRNA induced by hyperoxia is due to increased transcription or increased stability of mRNA.

Hyperoxia disrupts the normal oxidant-antioxidant balance in lung cells because of a markedly increased rate

Table 2 Lung AOE activity levels of premature rat after exposure to >95% oxygen or room air for seven days

		Hyperoxia group	Room air group	P
SOD	U/mgDNA	30.65±1.55	20.10±0.98	<0.001
GP	U/mgDNA	1.48±0.68	1.02±0.41	0.168
CAT	U/mgDNA	164.07±73.38	136.18±62.76	0.481

Values are expressed as mean±SD. N=7 samples in each group

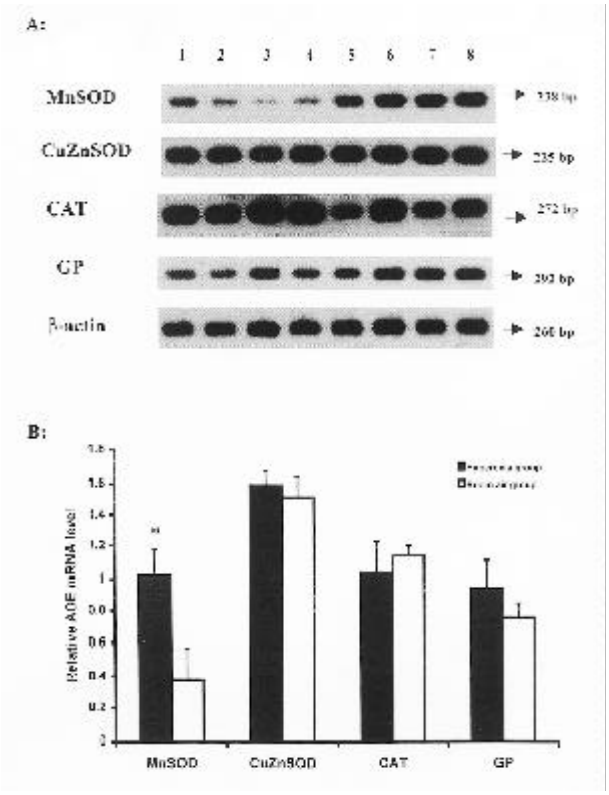

Figure 1 Assay of mRNA levels of AOE in the lungs of premature rats after exposure to >95% oxygen or room air for seven days (A): Southern blot hybridization analysis of RT-PCR products for MnSOD, CuZnSOD, CAT, GP and β-actin (internal control). Bands 1-4 were obtained from the room air group and bands 5-8 from the hyperoxia group. Lung tissues of 4 pups were studied in each group. (B): Relative amount of AOE gene transcripts, as expressed by the densitometry ratio of AOE to β-actin. Values are mean±SD. The hyperoxia group had a significantly higher level of MnSOD mRNA than those of the room air group (*p=0.0024). CuZnSOD, CAT, GP mRNA expressions were not significantly different between the two groups (p=0.3615, p=0.3465, and p=0.1258 respectively).

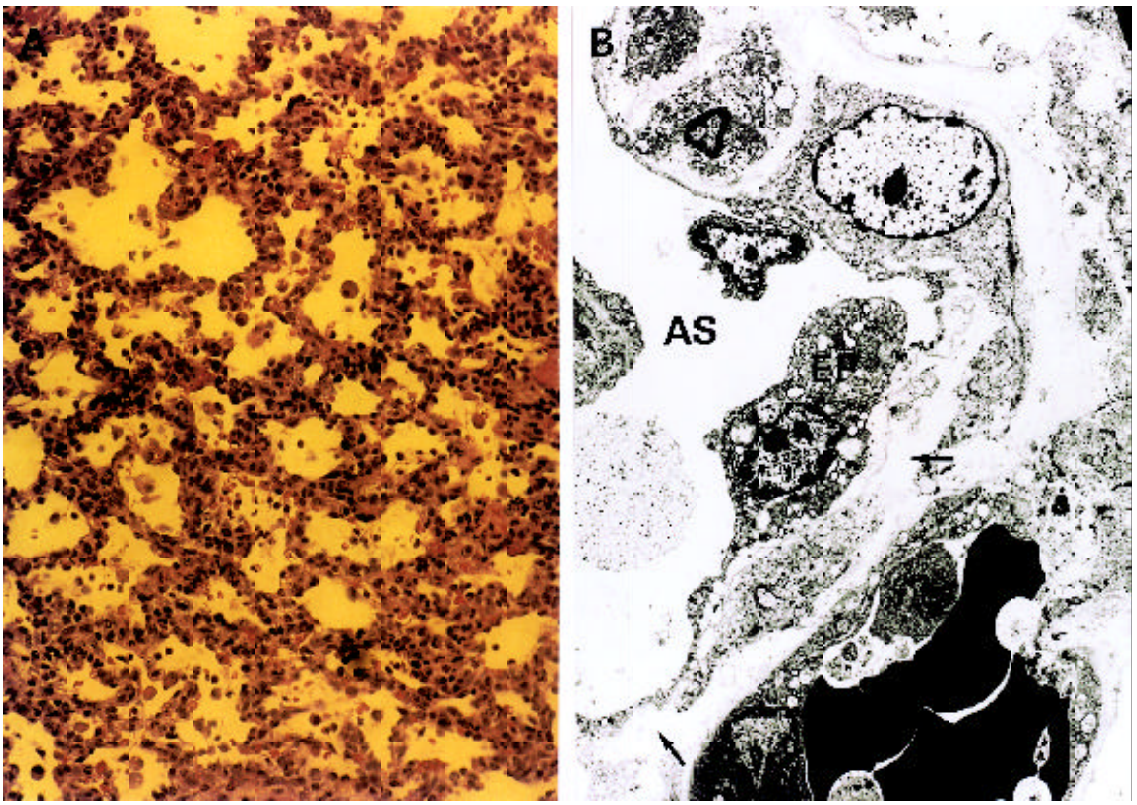

Figure 2 (A) Light micrograph (hematoxyline-eosin staining, magnification x 160) of a lung section of a preterm rat after seven days of exposure to > 95% oxygen, showing the presence of pulmonary edema, hemorrhage, disruption of alveolar structure, and formation of hyaline membrane. (B) Electron micrograph (magnification x 1650) of a lung section of the same animal, showing the presence of red blood cells and fibrin material within the airspace (AS), and degenerated type II epithelial cell (EP) loosely attached to the alveolar wall. Prominent edema (arrows) is identified in the interstitium.

of oxygen free radical production. The more rapidly and more completely this oxidant-antioxidant imbalance can be restored to normal by increasing the endogenous antioxidant defenses, the more effectively oxygen induced lung injury can be ameliorated. Our finding suggests that the preterm rat is capable of launching a protective response to hyperoxia-induced lung injury by preserving or increasing the production of SOD mRNA and SOD enzymes.[18] Compared to SOD, changes in CAT and GP activities and gene expression, if any, were relatively small although there was a trend showing that the activities of both enzyme levels increased at the same time. This suggests that the alteration of the gene expression of the various protective AOE by hyperoxia may be not coordinately regulated.[19]

The mechanism of hyperoxia in upregulating SOD activity and gene expression is not clearly understood. It has been postulated that proliferation of mitochondria-rich type2 epithelial cells is an important factor, since the mitochondrion is a major site of SOD production.[3] Recent studies have shown that various cytokines can rapidly activate the expression of the SOD gene in human, rat cells. Therefore it is possible that cytokines generated during lung inflammation induced by hyperoxia may play an important role in regulating SOD gene expression.[20] Since our measurement of mRNA was performed on total lung extracts, it is unclear whether the change is a generalized phenomenon throughout the various cell types present in the lung or a selective response of particular cell types. To unravel these questions will require detailed morphological studies using in situ hybridization and immunolocalization of AOE.

In conclusion, we have confirmed that like full term newborn animal, premature rats are able to increase SOD gene expression and enzyme activities in lung in exposure to hyperoxia. We also compared responses of premature versus full-term rat to prolonged hyperoxia (14 days). Results showed that preterm rat had relatively higher mortality, lower AOE activities than full-term rat (other report). The finding suggests that the prematurely born would be compromised under hyperoxic conditions and prone to develop rapid-onset oxygen toxicity, which might help explain why in human infants it is the more prematurely born who are most prone to develop early hyperoxic lung damage and BPD. Further studies are required to determine the mechanism on how this occurs and also the effect of hyperoxia on cellular enzyme of the AOE system.

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