Tolerance of Rats to Hyperoxia

Lung Antioxidant Enzyme Gene Expression

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Abstract

Tolerance to hyperoxia usually requires an increase of lung antioxidant enzyme (AOE) activity. We used rats with different degrees of tolerance to > 95% O_2 to evaluate the importance of individual AOEs for tolerance; we also explored the regulation of AOE gene expression. During exposure of adult rats to > 95% O_2, lung manganese superoxide dismutase (MnSOD) activity fell ~ 50% despite a threefold increase of MnSOD mRNA concentration; addition of a reducing agent to lung extracts from O_2-exposed rats partially restored MnSOD activity. Endotoxin induced tolerance to O_2 (*a*) without elevating Cu,Zn superoxide dismutase activity, (*b*) with increases of catalase and glutathione peroxidase (GP) activity of the same magnitude as occurred in O_2-saline rats, but (*c*) with MnSOD activity 1.5–1.9-fold higher than in air-saline rats and 1.4–3.6-fold higher than in O_2-saline rats. Endotoxin elevated the concentration of MnSOD and GP mRNAs without increasing their stability. O_2 elevated MnSOD mRNA concentration, and increased its stability. O_2 plus endotoxin increased the concentration and stability of MnSOD, catalase, and GP mRNAs. These data suggest that in adult rats tolerance to hyperoxia requires increased MnSOD activity; the data show gene expression and regulation vary among the AOEs, and that increased stability of the AOEs' mRNAs plays an important role in AOE gene expression and in tolerance to hyperoxia. (*J. Clin. Invest.* 1993. 91:499–508.) Key words: catalase • copper, zinc superoxide dismutase • enzyme inactivation • glutathione peroxidase • manganese superoxide dismutase • messenger RNA stability

Introduction

Exposure of cells to oxygen at pressures higher than normally experienced causes damage to the cells' molecular components that can result in cellular dysfunction and death. In organisms that normally breathe air, the lung is the site of most damage during exposure to hyperoxia at 1 atm (1, 2). Death under these conditions is due to inadequate gas exchange; the latter is the consequence of filling the lung's airspaces and the pleural cavity with fluid as a result of disruption of the alveolar–capillary interface (3). When otherwise unmanipulated adult rats are exposed to > 95% O_2, very little lung edema or pleural effusion develop during the first 48 h but both increase greatly between 48 and 60 h; rats begin to die at ~ 60 h of exposure and most are dead by 72 h (4–7).

Current evidence indicates the damaging effects of oxygen are mediated by superoxide, hydrogen peroxide, and possibly the hydroxyl radical, moieties whose cellular production increases during hyperoxia (8–10). The ability to resist the potentially damaging effects of oxygen (termed tolerance) varies among cells (11, 12) and between species (13). Tolerance can be age-dependent (13–15) and enhanced experimentally (16–20) and pharmacologically (4, 21). In most cases, age-dependent tolerance (13, 14) and tolerance induced pharmacologically (4, 21) or by experimental manipulation (4, 6, 16, 20, 22) are associated with an elevation of lung antioxidant enzyme (AOE)[1] activity during exposure. However, there is little information on the relative importance of individual AOEs to the lung's ability to tolerate hyperoxia (4, 23, 24). In addition, many prior studies that have demonstrated an association between tolerance to O_2 and an increase in lung AOE activity have not included measurements of manganese superoxide dismutase (MnSOD) activity (4–7, 13, 14, 20–22). MnSOD is encoded on nuclear genes but exhibits its activity in mitochondria (25); it may be especially important for tolerance in view of the evidence that cell death during exposure to hyperoxia correlates with loss of mitochondrial function as indicated by the inactivation of key mitochondrial enzymes, a fall in cellular O_2 consumption, and a decrease in cellular ATP concentration (26).

We have now taken advantage of age-dependent differences in tolerance to O_2 (13–15), and of the ability to induce tolerance to O_2 by treatment with endotoxin (4, 21), to determine in rats if there are correlations between the lung activity of individual AOEs and the development of lung edema during exposure to > 95% O_2. We have also examined the level at which lung AOE gene expression is regulated in response to O_2, endotoxin, and O_2 plus endotoxin. Our findings support a conclusion that, in adult rats, lung MnSOD is of particular importance to the induction of tolerance to O_2. Additionally, we show that AOE gene expression and regulation varies among the enzymes in response to O_2, endotoxin, and O_2 plus endotoxin and that increased AOE mRNA stability plays an important role in AOE gene expression in rats made tolerant to O_2 by endotoxin.

Methods

Animals. We used specific pathogen-free male Sprague Dawley rats obtained from Zivic Miller Labs Inc. (Allison Park, PA) or from Harlan Farms (Indianapolis, IN). They were maintained in our Animal

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1. *Abbreviations used in this paper:* AOE, antioxidant enzyme; CAT, catalase; DDC, diethyldithiocarbamate; GP, glutathione peroxidase; TBAR, thiobarbituric acid–reactive material.

Care Facilities on a 12-h light-dark cycle and were allowed food (Rodent Laboratory Chow 5001, Ralston-Purina Co., St. Louis, MO) and water ad libitum.

Exposures and treatments. All rats were exposed to > 95% O_2 at 1 atm, or to air from a compressed-air generator, in identical 3.5-ft^3 chambers constructed from clear plastic. The other conditions of exposure were < 0.1% CO_2, 22–25°C, and 40–60% humidity. Exposures were continuous for the times indicated except for 10–15 min daily when the chambers were opened for housekeeping purposes or to allow us to inject animals with endotoxin or its diluent.

We used lipopolysaccharide (endotoxin) from *Salmonella typhimurium* (Sigma Chemical Co., St. Louis, MO). Rats were injected intraperitoneally immediately before exposure (time zero), and in some experiments 24 or 36 h later, with endotoxin (500 $\mu g \cdot kg^{-1}$) in 0.15 M NaCl (saline) at a concentration of 100 $\mu g \cdot ml^{-1}$. Rats not given endotoxin received an equal volume of saline intraperitoneally. Rats were anesthetized (pentobarbital sodium, ~ 80 $mg \cdot kg^{-1}$) and killed by cutting the great vessels of the abdomen.

For experiments in which we measured lung weights, the left lung was removed without having been perfused. For all other measurements the lungs were perfused via the pulmonary artery with 20 ml of ice-cold saline. The perfused lungs were then excised, frozen in liquid nitrogen, and immediately processed for the various assays or stored in a freezer at −70°C for later use.

Assays for enzyme activity. To determine the activity of the SODs ~ 600 mg of lung tissue was disrupted in 6 ml of 5 mM KPi for 50 s with a Polytron Homogenizer P110/35 with a 10-mm-diameter generator (Brinkmann Instruments, Inc., Westbury, NY) operated at its highest speed. The homogenate was centrifuged at 27,000 *g* for 45 min at 0–4°C. The supernatant material was dialyzed overnight at 0–4°C against three changes of 40 vol 5 mM KPi with 0.1 mM EDTA. The xanthine oxidase-cytochrome *c* method was used for all assays of SOD (27), and sodium cyanide (0.015 mM) was used to inhibit cytochrome oxidase. A unit of SOD activity was the amount that halves the rate of reduction of cytochrome *c*. We used diethyldithiocarbamate (DDC) in a procedure that allows quantitation of SOD activity due to Cu,ZnSOD and MnSOD (28).

To measure catalase (CAT) and glutathione peroxidase (GP) activity ~ 400 mg of lung tissue was disrupted in 4 ml of potassium phosphate buffer (KPi) and the 27,000-*g* supernatant fraction was obtained as described above and assayed without having been dialyzed. CAT activity was measured as the rate of disappearance of H_2O_2 at 240 μm (29, 30). One unit of CAT activity decomposes 1 μmol of $H_2O_2 \cdot min^{-1}$ at 25°C and pH 7.0. GP activity was measured by the GSH-GSSG reductase recycling method (31) using cumene hydroperoxide as substrate. One unit of GP activity oxidizes 1 μmol NADPH $\cdot min^{-1}$. An editorial reviewer of our manuscript for this article correctly pointed out that cumene hydroperoxide is a substrate for glutathione-S-transferase as well as for GP activity (32). We therefore did additional exposures and assayed GP activity using hydrogen peroxide, which does not serve as a substrate for glutathione-S-transferase (32).

During the editorial review of the manuscript a question was raised regarding the use of the 27,000-*g* supernatant fraction rather than the whole homogenate; the concern was that some enzyme activity might be lost by sedimentation of undisrupted lung cells, and, if this occurred unequally in our experimental groups, it might influence our results. To test this possibility we exposed rats to air or O_2 for 48 h. The groups were air-saline, air-endotoxin, O_2-saline, and O_2-endotoxin as described in the legend to Table 5. Lung tissue was homogenized as described above, i.e., 50 s with a Polytron Homogenizer operated at its highest speed. A sample of this homogenate was taken. The remaining homogenate was homogenized for an additional minute (a total of 1 min 50 s) and a sample was taken from this homogenate. The remaining homogenate was homogenized for an additional minute (a total of 2 min 50 s) and a sample taken. Finally, the remaining homogenate was homogenized for two additional minutes (a total of 4 min 50 s). All samples were centrifuged at 27,000 *g* for 45 min and the supernatant material assayed for catalase activity. We chose catalase because it is located in two organelles (peroxisome and mitochondria) (33) and hence would be most representative of potentially unruptured cellular compartments. We found that prolonging the homogenization increased catalase activity in the supernatant fraction but did so in a uniform manner and so does not alter our conclusions. For air-saline lungs (n = 3), the percent increase in catalase activity at 1 min 50 s, 2 min 50 s, and 4 min 50 s, compared to the 50-s value, was 11.4±7, 18.2±9.2, and 11.3±3.6. For air-endotoxin lungs (n = 4) the respective percent increases were 10.3±4.1, 21.6±1.7, and 15.1±5.5. For O_2-saline lungs (n = 3), the respective percent increases were 13.3±3.3, 7.4±1.4, and 6.0±3.9. For O_2-endotoxin lungs (n = 4), the respective percentage increases were 19.9±5.7, 21.8±3.1, and 31.5±8.4. $P > 0.05$ between groups at each duration of homogenization by analysis of variance. Thus, although additional homogenization released more enzyme, it did so in a manner that does not influence our intergroup comparisons.

In vitro experiments with dithiothreitol (DTT). We examined the effect on the activity of Cu,ZnSOD and MnSOD of in vitro incubation of the reducing agent, DTT, with lung extracts. Lungs from otherwise untreated rats exposed to air or > 95% O_2 for 48 h were perfused and excised, and the 27,000-*g* supernatant fraction was isolated. This fraction was dialyzed as described above and was then divided into two samples. One sample was incubated with DTT at 25°C for 30 min and the other sample was incubated in an identical manner without the reagent but with an equal volume of water (the diluent for DTT). Both samples were then dialyzed overnight at 0–4°C against 3,000 vol of 5 mM KPi that was changed four times. The dialyzed extracts were assayed for Cu,ZnSOD and MnSOD activity. Because we compared the effect of DTT on MnSOD activity and Cu,ZnSOD activity to their activity in lung extract without DTT, i.e., the same extract with and without DTT, activity was expressed per milligram of protein (Table 10) rather than per milligram of DNA.

cRNA preparation. A pGEM-Blue construct containing the 3′ end of an α-actin cDNA was a gift from Dr. Rudolf K. Werner (Department of Biochemistry and Molecular Biology, University of Miami School of Medicine, Miami, FL) and was used to synthesize actin [^{3}H]-cRNA for use as a mRNA recovery marker (34). A rat liver catalase cDNA, PMJ1010, was a gift from Dr. Shuichi Furuta (Shinshu University School of Medicine, Nagano, Japan). The catalase cDNA insert in this pBR322 plasmid contains a 1,133-basepair Pst I restriction endonuclease fragment corresponding to 21 bases of 5′ untranslated cDNA and 1,112 bases of sequence coding for CAT amino acids 1–370. This fragment was subcloned into pGEM-3Z, and was used to generate a ^{35}S-labeled antisense cRNA and standard sense cRNA as previously described in detail (35).

We obtained a rat liver GP cDNA, pGPX1211, from Dr. Ambati Reddy (Pennsylvania State University, University Park, PA). This plasmid contains the complete coding sequence for GP as well as 36 basepairs of 5′ and 930 basepairs of 3′ untranslated sequence. We ligated the 534-basepair Eco RI restriction endonuclease fragment, corresponding to bases 318–850, into pGem-7Z vector (Promega Corp., Madison, WI). This insert contains the coding region for amino acid residues 90–200 as well as 204 bases of 3′ noncoding sequence and was used to generate ^{35}S-labeled antisense cRNA probe and sense cRNA standard as previously described (35).

We obtained a rat liver MnSOD cDNA from Dr. Ye-Shih Ho (Duke University, Durham, NC), subcloned a EcoRI-StuI restriction fragment of this cDNA into pGEM-3Z vector, and sequenced the insert to be able to transcribe a MnSOD cRNA ^{35}S-labeled antisense probe and sense-strand standard. This 533-base fragment includes 11 bases of 5′ noncoding region, 72 bases of the sequence for the leader peptide responsible for transport into mitochondria, and 450 bases of coding region corresponding to amino acids 1–150.

Solution hybridization. The concentration of mRNA was measured in total nucleic acids isolated from the lung (35) using the method of Durnam and Palmiter (36). We added [^{3}H]actin cRNA at the start of the process to isolate total RNA to enable us to account for mRNA lost during the extraction process (34).

mRNA degradation. The rates of degradation of MnSOD mRNA, CAT mRNA, and GP mRNA were measured in vitro (35). Excised lungs were sliced with a McIlwain tissue chopper (Brinkmann Instruments, Inc.) set to make 1.0-mm slices. The slices (~ 1 g) were incubated at 37°C at a shaking frequency of 120 cycles/min in 20 ml of Krebs-Ringer bicarbonate medium ([in mM] 118 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 2.5 $NaHCO_3$) pH 7.4, containing 5.5 mM glucose, adult rat serum concentrations of 20 L-amino acids (37), and 10 μg/ml actinomycin D. In all experiments the medium was equilibrated with CO_2-O_2 (1:19) before use and the incubations were carried out in a gas phase of CO_2-O_2 (1:19). At 0, 3, 6, and 9 h of incubation ~ 0.25 g of tissue were removed from the flask, quickly washed with 0.15 M NaCl, and frozen by immersion in liquid nitrogen. The tissue samples were stored at −70°C until total nucleic acids were isolated and assayed for the amount of MnSOD mRNA, CAT mRNA, and GP mRNA as described above. Half-lives were calculated from the slope of the first order decay curves.

Chemical analysis. DNA was measured using calf thymus DNA as standard (38). Thiobarbituric acid–reactive material (TBAR) was assessed after the hydrolysis of lung extracts under acid conditions (39). Malondialdehyde was used as a standard. Protein was measured using Coomassie Plus assay reagent from Pierce Chemical Co. (Rockford, IL) with bovine serum albumin as the standard.

Lung weights. Lung wet weight was measured in unperfused lung tissue that had been blotted dry on its external surface and then cut into small pieces. The tissue was placed in an oven at 80°C until two weights, 24 h apart, were unchanged; this represented the dry weight.

Data collection and statistical analysis. For each parameter measured, or calculated from measurements, the values for individual animals were averaged per experimental group and the SE of the group mean was calculated. The significance of the difference between two means was determined by an unpaired *t* test analysis (40) except for the data in Table 10 where we used a paired *t* test analysis. The significance of differences between more than two treatments was determined by an analysis of variance (41, 42). We expressed all enzyme activity and mRNA amount per milligram of DNA except the data in Table 10. DNA was chosen as a reference substance because serum proteins leak into the lung in O_2-saline rats and would therefore falsely elevate what would be taken as lung protein. In Table 10 we compare the effect of DTT on the enzyme activity of a given sample. That is, in lung extracts from "air" rats we measured MnSOD activity in the presence of 0, 1.5, and 2.5 mM DDT; the comparison was among these three concentrations of DTT. Similar, in lung extracts from "O_2" rats we measured MnSOD activity in the presence of 0, 1.5, and 2.5 mM DTT; the comparisons were again among these three concentrations of DTT. In that the question was not an effect of O_2 on enzyme activity compared to activity in air rats, but the effect of DTT on activity in a given extract, relating activity to protein is appropriate.

Results

Time course of AOE activity, lung edema, and pleural fluid during exposure of otherwise untreated rats to > 95% O_2. Cu,ZnSOD activity did not exhibit intergroup (air vs. O_2) differences at any time (Table I). MnSOD activity was the same in air-exposed and O_2-exposed rats at 24 h but was ~ 40% lower in O_2 rats than in air rats at 48 and 60 h (Table I). CAT activity was greater in O_2 rats than in air rats at each time and the difference increased as the duration of exposure was prolonged (Table I). GP activity was higher in O_2 rats than air rats at 24, 48, and 60 h; the difference was largest at 60 h.

There was evidence of increased lung water in O_2 rats at 48 h with relatively little pleural fluid (Table II). The ratio of left lung wet weight to initial body weight was 43% greater in O_2 rats than in air rats at 48 h and 73% greater at 60 h. The ratio of lung wet to dry weight was 16% higher in O_2 rats than in air rats at 48 h and 21% higher at 60 h. The most striking evidence of damage was the ~ 20-fold increase in the volume of pleural fluid that developed in O_2 rats between the 48th and 60th h of exposure.

Age-dependent response of AOE activity and lung weight during exposure of otherwise untreated rats to > 95% O_2 for 48 h. Age and body weight are closely related in rats (43) and young rats are more tolerant of hyperoxia than old rats (13–15). We therefore chose rats of clearly different body weight to compare the lung's AOE response to hyperoxia to the magnitude of O_2-induced lung damage as revealed by indicators of lung edema. Cu,ZnSOD activity did not exhibit intergroup (air vs. O_2) differences at any age (Table III). In the youngest group

Table I. Time Course of AOE Activity during Exposure to > 95% O_2

Exposure	Duration	AOE activity			
		Cu,ZnSOD	MnSOD	CAT	GP
	h	*units · mg⁻¹ DNA*			
Air	24	71.5±3.0 (6)	7.3±0.8 (6)	100±7 (7)	1.5±0.1 (7)
O_2	24	70.6±2.8 (6)	7.6±0.7 (5)	118±4 (7)	1.9±0.1 (7)
P values		NS	NS	< 0.05	< 0.001
Air	48	86.7±4.7 (4)	10.1±0.7 (8)	110±4 (8)	1.6±0.0 (8)
O_2	48	99.2±7.9 (4)	5.2±0.4 (8)	147±7 (8)	2.0±0.1 (8)
P values		NS	< 0.001	< 0.01	< 0.001
Air	60	92.1±3.8 (8)	11.2±0.5 (8)	115±4.0 (8)	1.7±0.0 (8)
O_2	60	92.1±5.1 (8)	6.8±0.3 (8)	189±8.0 (8)	2.5±0.1 (8)
P values		NS	< 0.001	< 0.001	< 0.001

Adult male rats were kept in chambers at an O_2 concentration of 20.9% (Air) or > 95% O_2 (O_2) for 24, 48, or 60 h. Mean±SE are given. Figures in parentheses indicated the number of rats. NS = $P > 0.05$. The assays of enzyme activity for air-exposed rats and O_2-exposed rats were done simultaneously for each period of exposure but the assays for the different periods of exposure were not performed simultaneously. Thus, the differences in air rats of Cu,ZnSOD and MnSOD activity at 24, 48, and 60 h reflect day-to-day variability in the assay. The initial body weights of the rats exposed to air for 24, 48, and 60 h were, respectively, 305±5.4, 407±4.9, and 292±5.0 g; for O_2 rats at 24, 48, and 60 h the weights were 316±7.5, 345±22, and 283±4.5 g.

Table II. Lung and Pleural Fluid during Exposure to > 95% O_2

Exposure	Duration	Left lung/ body wt	Lung wet/dry wt	Pleural fluid
	h	*g/kg initial body wt*		*ml*
Air	48	1.4±0.1 (9)	5.1±0.0 (9)	None obtained (9)
O_2	48	2.0±0.1 (9)	5.9±0.1 (9)	0.4±0.2 (9)
P values		< 0.001	< 0.001	
Air	60	1.5±0.1 (8)	5.2±0.0 (8)	None obtained (8)
O_2	60	2.6±0.1 (5)	6.3±0.1 (5)	7.7±0.9 (5)
P values		< 0.001	< 0.001	

Adult male rats were exposed as explained in the legend to Table I. Mean±SE are given. Values in parentheses indicate the number of rats.

(~ 140 g of body weight) MnSOD activity of air rats and O_2 rats was not different; in rats that weighed ~ 280 g MnSOD activity was ~ 40% lower in O_2 rats than in air rats, and in the rats that weighed ~ 435 g MnSOD activity was ~ 45% lower in O_2 rats than in air rats. CAT activity was greater in O_2 rats than in air rats at each age and the difference increased with age, 37%, 59%, and 71%, respectively, in the youngest, middle, and oldest groups. GP activity was greater in O_2 rats than in air rats in the two groups of older rats but the intergroup differences were unchanged with age.

Exposure to 48 h of > 95% O_2 had a greater affect on indicators of lung edema in older rats than in younger rats: the ratio of left lung wet weight to initial body weight was 14%, 36%, and 64% greater in O_2 rats than air rats in the youngest, middle, and oldest groups, respectively; similarly, O_2 exposure induced a greater increase in the wet to dry weight ratio in older than in younger rats (Table IV). Rats in the youngest group exposed to O_2 gained weight at a slower rate than air-exposed rats in the same age group. Older rats stopped growing or lost weight during exposure to O_2 (Table IV). The fall in the ratio of lung weight to body weight evident with increasing age in air rats (Table IV) is a normal occurrence (43).

AOE activity, lung edema, pleural effusion, and TBAR in saline-treated and endotoxin-treated rats exposed to air or > 95% O_2. In the next series of experiments we took advantage of the ability of endotoxin treatment to induce tolerance in rats to hyperoxia (4–6, 21) to further evaluate the temporal relation between lung AOE activity and the development of lung edema. Cu,ZnSOD activity did not exhibit intergroup differences (air vs. O_2) at 24, 48, or 60 h of exposure (Table V). MnSOD activity was higher in endotoxin-treated than in saline-treated rats regardless of the duration of exposure. However, irrespective of the length of exposure, air-endotoxin rats had higher MnSOD activity than O_2-endotoxin rats. MnSOD activity in O_2-saline rats at 24 h of exposure was the same as in air-saline rats but was 32% lower at 48 h and 55% lower at 60 h. CAT and GP activities were not elevated by endotoxin treatment alone, and, importantly, were equally elevated in O_2-saline and O_2-endotoxin rats at each time. As found in uninjected rats (Table I), the difference in CAT and GP activity between air-saline and O_2-saline rats became larger as the duration of exposure increased (Table V).

In the experiments whose results are presented in Table V, we used cumene hydroperoxide as the substrate in our assays for GP activity. This hydroperoxide also serves as a substrate for glutathione-S-transferase (32) and hence our findings on GP could have been influenced by the activity of glutathione-S-transferase. We therefore undertook additional experiments. We exposed rats for 48 h exactly as described in Table V and assayed GP using H_2O_2 as substrate; glutathione-S-transferase does not act on H_2O_2 (32). We found that GP activities ($U \cdot mg^{-1}$ DNA) for air-saline, air-endotoxin, O_2-saline, and O_2-endotoxin rats were, respectively, 1.3±0.1 ($n = 6$), 1.4±0.1 (7), 2.3±0.1 (6), 2.2±0.2 (4). The values for both O_2 groups were significantly greater than the values for both air groups ($P < 0.05$). These are virtually identical to those at the same time (48 h) in Table V; this indicates that our data using cumene hydroperoxide as substrate reflect GP activity.

As previously shown (4), treatment of O_2-exposed rats with endotoxin resulted in a smaller increase in the lung's wet to dry weight ratio and in marked protection against the development of pleural effusion (Table VI). Endotoxin treatment did not

Table III. Age-dependent Response of Lung AOE Activity to > 95% O_2

Exposure	Body wt	AOE activity			
		Cu,ZnSOD	MnSOD	CAT	GP
	g	*units · mg^{-1} DNA*			
Air, 48 h	137±5 (11)	64.1±2.0 (4)	10.7±1.5 (8)	139±5 (8)	1.2±0.0 (8)
O_2, 48 h	145±4 (11)	71.1±6.0 (4)	10.0±1.3 (8)	191±7 (8)	1.3±0.1 (8)
P values	NS	NS	NS	< 0.001	NS
Air, 48 h	279±6 (10)	72.2±5.2 (4)	19.1±0.8 (4)	102±5 (8)	1.5±0.1 (8)
O_2, 48 h	289±6 (10)	68.0±3.4 (4)	11.3±1.4 (4)	163±6 (8)	2.0±0.1 (8)
P values	NS	NS	< 0.005	< 0.001	< 0.01
Air, 48 h	436±7 (7)	73.3±6.3 (4)	23.2±1.3 (6)	107±0.4 (8)	1.6±0.1 (8)
O_2, 48 h	437±8 (10)	62.1±1.8 (4)	12.6±0.9 (8)	183±5.0 (8)	2.1±0.1 (8)
P values	NS	NS	< 0.001	< 0.001	< 0.005

Male rats of the initial average weight shown were exposed to air or > 95% O_2. Mean±SE are given. Values in parentheses indicate the number of rats. NS = $P > 0.05$. The assays of enzyme activity were done simultaneously for each weight group; the differences in air-exposed rat activity for each enzyme therefore are age-related changes; similar findings have been reported before for Cu,ZnSOD (68) and for CAT and GP (69).

Table IV. Age-dependent Response of Body Weight and Lung Weight to > 95% O_2

Exposure	Body wt		Lung edema	
	Initial	Final	Left lung/ body wt	Left lung wet/dry wt
	g		*g/kg initial body wt*	
Air, 48 h	137±5 (11)	157±5 (11)	2.1±0.1 (11)	5.1±0.0 (11)
O_2, 48 h	145±4 (11)	151±4 (11)	2.4±0.1 (10)	5.9±0.1 (10)
P values	NS	NS	< 0.005	< 0.001
Air, 48 h	279±6 (10)	298±6 (10)	1.4±0.1 (8)	5.1±0.0 (9)
O_2, 48 h	289±6 (10)	287±7 (10)	1.9±0.1 (9)	5.9±0.1 (9)
P values	NS	NS	< 0.005	< 0.001
Air, 48 h	436±7 (7)	446±7 (10)	1.1±0.0 (10)	5.0±0.0 (10)
O_2, 48 h	437±8 (10)	429±7 (10)	1.8±0.1 (9)	6.1±0.1 (10)
P values	NS	NS	< 0.001	< 0.001

Exposures are as explained in the legend to Table I. Initial and final body weight refer, respectively, to weight immediately before the onset of exposure and at 48 h of exposure. Values in parentheses indicate the number of rats. NS = $P > 0.05$.

affect the lung concentration of TBAR in rats that continued to breathe air (Table VII). However, O_2-saline rats exhibited a 46% increase in concentration of TBAR; this increase was absent in O_2-endotoxin rats.

AOE mRNA concentration and half-life. The concentration of MnSOD mRNA was elevated in air-endotoxin rats, O_2-saline rats, and O_2-endotoxin rats (Table VIII); the elevation in O_2-exposed rats was greater than in air-endotoxin rats. Neither oxygen nor endotoxin alone altered the concentration of CAT mRNA but the combination of O_2 plus endotoxin resulted in an ~ 50% increase in the concentration of CAT mRNA (Table VIII). Endotoxin alone increased the concentration of GP mRNA, oxygen alone did not, and the combination of O_2 plus endotoxin increased the concentration of GP mRNA, but the values for air-endotoxin and O_2-endotoxin rats were not significantly different from each other (Table VIII).

The half-life of MnSOD mRNA was not altered by endotoxin alone but it was lengthened to an equal degree in O_2-saline and O_2-endotoxin rats (Table IX). Neither endotoxin alone nor O_2 alone altered the half-life of CAT mRNA or GP mRNA. Only the combination of O_2 and endotoxin increased the half-life of CAT mRNA and of GP mRNA (Table IX).

In vitro recovery of MnSOD activity. The fall in MnSOD activity during exposure of otherwise untreated rats (Tables I and III) and saline-treated rats (Table V) to oxygen, in association with an elevated concentration of MnSOD mRNA (Table VIII), led us to consider the possibility that oxidation of components of the enzyme resulted in its partial inactivation. We therefore performed experiments in which we treated lung extracts from rats exposed to air or to > 95% O_2 for 48 h with the reducing agent DTT. This treatment did not elevate Cu,ZnSOD activity or MnSOD activity in lung extracts from air-exposed rats nor Cu,ZnSOD activity in lung extracts from O_2-exposed rats; it did increase MnSOD activity in lung extracts from O_2-exposed rats (Table X).

Discussion

AOEs and tolerance to oxygen in rats. Most evidence, especially in mammalian systems, that AOEs protect against the

Table V. Time Course of AOE Response to > 95% O_2 in Saline-treated and Endotoxin-treated Rats

Conditions	Exposure	AOE activity			
		Cu,ZnSOD	MnSOD	CAT	GP
	h	*units · mg⁻¹DNA*			
Air-saline	24	95.0±3.8 (7)	10.1±0.6 (8)	186±28 (8)	1.7±0.0 (3)
Air-endotoxin	24	91.4±2.7 (8)	21.9±2.0 (7)	191±21 (8)	1.7±0.0 (3)
O_2-saline	24	100.8±3.5 (8)	12.9±1.2 (8)	223±32 (8)	1.9±0.2 (3)
O_2-endotoxin	24	101.1±3.0 (7)	19.0±1.2 (7)	230±28 (8)	1.9±0.0 (4)
Air-saline	48	111.3±5.6 (8)	12.4±0.4 (8)	193±11 (8)	1.7±0.1 (3)
Air-endotoxin	48	110.1±5.8 (8)	21.1±2.3 (8)	199±10 (8)	1.6±0.1 (4)
O_2-saline	48	119.0±10.3 (8)	8.4±0.4 (8)	267±11 (8)	2.0±0.1 (3)
O_2-endotoxin	48	104.0±14.8 (8)	18.9±0.8 (8)	262±16 (8)	2.0±0.1 (4)
Air-saline	60	88.3±3.6 (4)	9.3±0.3 (4)	114±10 (4)	1.0±0.0 (4)
Air-endotoxin	60	80.4±4.4 (4)	25.6±1.3 (4)	108±3 (4)	1.0±0.0 (4)
O_2-saline	60	78.3±4.1 (4)	4.2±0.3 (4)	181±6 (4)	1.5±0.1 (4)
O_2-endotoxin	60	81.7±7.9 (4)	15.2±2.6 (4)	180±14 (4)	1.4±0.1 (4)

Adult male rats were injected intraperitoneally with *S. typhimurium* lipopolysaccharide (endotoxin) (500 $\mu g \cdot kg^{-1}$) in phosphate-buffered 0.15 M NaCl (saline) at zero time; in rats exposed for 48 h a second injection was given 24 h after the zero time injection; in rats exposed for 60 h the second injection was given 36 h after the zero time injection. Saline injected rats received equal volumes of saline on the same schedule. Rats were exposed to air or > 95% O_2 for the times indicated. Mean±SE are given; values in parentheses indicate the number of rats. For Cu,ZnSOD $P > 0.05$ for each intragroup mean at each time. For MnSOD at 24 h $P > 0.05$ for air-saline vs. O_2-saline and for air-endotoxin vs. O_2-endotoxin, $P < 0.05$ for each saline vs. each endotoxin mean; at 48 h $P < 0.05$ between each mean; at 60 h $P < 0.05$ between each mean. For CAT and GP at each time, O_2-saline and O_2-endotoxin $P < 0.05$ vs. air-saline and air-endotoxin but $P > 0.05$ between O_2-saline and O_2-endotoxin. The initial body weights of the 24-h air-saline, air-endotoxin, O_2-saline, ar O_2-endotoxin were respectively: 283±6.2, 283±3.6, 280±3.6, and 294±2.8 g. For 48-h rats the initial body weights given in the same sequence were 298±4.3, 295±7.4, 303±4.5, and 297±7.0 g. For the 60-h rats the initial body weight in the same sequence were 307±1.1, 314±3.7, 310±3.8, and 312±5.7 g.

Table VI. Endotoxin, Lung Weight, and Pleural Effusion

Conditions	Exposure	Wet/dry wt	Pleural fluid
	h		*ml*
1. Air-saline	60	5.1±0.0 (4)	ND (4)
2. Air-endotoxin	60	4.9±0.0 (3)	ND (4)
3. O_2-saline	60	6.7±0.2 (6)	4.9±1.0 (6)
4. O_2-endotoxin	60	5.5±0.0 (4)	ND (4)
P values	1 vs. 2	NS	
	1 vs. 3	< 0.01	
	1 vs. 4	< 0.05	
	2 vs. 3	< 0.01	
	2 vs. 4	< 0.05	
	3 vs. 4	< 0.05	

Adult rats were injected with endotoxin or saline and exposed to air or O_2 as described in the legend to Table V. Mean±SE are given. Values in parentheses indicate the number of rats. NS = $P > 0.05$; ND = none detected.

damaging effects of oxygen is correlative: an increase in AOE activity during exposure to hyperoxia, or an elevation produced before exposure and maintained during exposure, are associated with less O_2-induced damage and fewer fatalities than occur without elevated antioxidant enzyme activity (4, 13, 16, 22, 44). More direct evidence for a protective effect of AOEs comes from genetic manipulations that cause the presence or absence of an AOE and result, respectively, in increased or decreased tolerance to O_2 (45, 46). However, even studies in which cells have been transfected with the gene for a specific AOE, and overproduce the enzyme, have not always provided an unambiguous indication of the enzyme's physiological importance to the cell's response to an oxidant stress. For example, elevated SOD activity in the presence of an oxidant stress would generate an increased amount of hydrogen peroxide that, in the absence of increased CAT or GP activity, could diminish tolerance (47). This supports the notion that protection against increased production of superoxide requires a balanced increase in AOEs, SOD to dismutate the excess superoxide and CAT or GP to eliminate the resultant additional H_2O_2 (48). Further increasing the complexity of the problem, cells transfected with a Cu,ZnSOD cDNA that overproduce the enzyme (47), and transgenic mice that overproduce Cu,ZnSOD (49), have increased GP activity that is presumably an adaptive response to the production of extra hydrogen peroxide; hence, in those experiments, it was not possible to produce an isolated increase in Cu,ZnSOD gene expression. Thus, even experiments utilizing techniques of genetic engineering, as those based on correlations between AOE activity and tolerance, may leave some uncertainty.

We now provide data that, taken with findings in the literature, are consistent with the notion that elevation of lung MnSOD activity is essential for lung tolerance to hyperoxia in adult rats. The most compelling evidence is the following: (*a*) Endotoxin treatment results in nearly 100% survival of adult rats during 72 h of exposure to > 95% O_2 compared to ~ 30% survival in untreated rats (4–7, 13, 14). This remarkably enhanced survival occurs with substantial protection against the development of lung edema and pleural effusion (Table VI and reference 4) and with the abrogation of evidence of increased lipid peroxidation (Table VII). (*b*) The only difference, with respect to lung AOE activity, between O_2-saline and O_2-endotoxin rats relates to MnSOD activity; the activity falls ~ 50% during exposure of O_2-saline rats and increases 200–350% during exposure of O_2-endotoxin rats (Table V). Cu,ZnSOD activity is not altered during 60 h by endotoxin, O_2, or endotoxin plus O_2 (Table V). CAT activity increases to the same extent and with the same timing in O_2-saline and O_2-endotoxin rats; the same is true for GP activity (Table V). (*c*) Among otherwise unmanipulated rats of different age, the extent of the fall in MnSOD activity during exposure to hyperoxia correlates with the greater lung edema and pleural effusion that occurs in the older rats (Tables III and IV). Such a correlation is not found for the other antioxidant enzymes; in fact, the rise in CAT and GP activity in untreated- or saline-treated O_2-exposed rats became greater with age even as tolerance fell.

Our conclusion regarding the primacy of MnSOD in adult rat lung tolerance to hyperoxia is not consistent with two prior observations. First, transgenic mice that constitutively overexpress Cu,ZnSOD are resistant to pulmonary damage by hyperoxia (49). However, we believe the conclusion that the tolerance to hyperoxia is due to elevated Cu,ZnSOD activity is open to question for the following reasons: (*a*) Protection against O_2 was found in 2.5- but not in 5.5-mo-old transgenic mice whereas the latter expressed lung Cu,ZnSOD activity that was two- to threefold greater than was expressed in the younger mice. (*b*) The younger mice, but not the 5.5-mo-old mice, constitutively overexpressed GP and glucose-6-phosphate dehydrogenase, and indeed, the authors acknowledge this may have contributed to the better survival of the young compared to older transgenic mice (49). (*c*) The transgenic mice did not constitutively overexpress MnSOD activity but MnSOD activity in mice exposed to hyperoxia was not reported (47). Recent work indicates that in otherwise unmanipulated adult mice lung MnSOD activity increases significantly during exposure to > 95% O_2 (50). Thus, it is possible that MnSOD activity increased during exposure to hyperoxia in the transgenic mice, and that this increase, in association with the elevated activity of GP, was responsible for the enhanced tolerance to O_2 that was ascribed to Cu,ZnSOD.

A second observation that argues against our notion of the primacy of MnSOD for lung tolerance to hyperoxia is the demonstration by Frank, Summerville, and Massaro (4) that treat-

Table VII. Malondialdehyde Concentration in Lung at 48 h Exposure

Conditions	TBAR
	nmol MDH · mg⁻¹DNA
Air-saline (4)	4.1±0.1
Air-endotoxin (3)	4.3±0.4
O_2-saline (4)	6.0±0.4
O_2-endotoxin (4)	4.4±0.2

Rats were given saline or endotoxin and exposed as described in the legend to Table V. Mean±SE are given. Values in parentheses indicate the number of rats. $P < 0.01$ between O_2-saline and each other mean; $P > 0.05$ between the other means. Abbreviation: MDH, malondialdehyde.

Table VIII. AOE mRNA Concentration

Condition	Time	AOE mRNA concentration		
		MnSOD	CAT	GP
	h	*molecules mRNA · mg⁻¹ DNA × 10⁴*		
1. Air-saline	48	2,476±191 (12)	4,275±541 (8)	5,008±690 (12)
2. Air-endotoxin	48	5,204±106 (11)	4,311±475 (8)	7,226±680 (12)
3. O_2-saline	48	7,458±904 (11)	4,008±454 (8)	5,818±661 (12)
4. O_2-endotoxin	48	9,572±1,344 (12)	6,225±866 (7)	9,061±764 (12)
P values	1 vs. 2	< 0.05	NS	< 0.01
	1 vs. 3	< 0.001	NS	NS
	1 vs. 4	< 0.001	< 0.01	< 0.01
	2 vs. 3	< 0.05	NS	NS
	2 vs. 4	< 0.001	< 0.01	NS
	3 vs. 4	NS	< 0.01	< 0.01

Rats were given saline or endotoxin and exposed as described in the legend to Table V. Mean±SE are given. Values in parentheses indicate the number of rats.

ment of rats with DDC, which inhibits Cu,ZnSOD but not MnSOD (28), substantially decreases endotoxin-induced tolerance. However, recent work showing that inhibition of erythrocyte SOD by DDC also depletes the cells of glutathione raises the possibility that the action of DDC previously attributed to its effect on Cu,ZnSOD was in fact due to its action on the concentration of glutathione in lung cells (51). The notion that Cu,ZnSOD plays a significant role in lung tolerance to hyperoxia induced by endotoxin treatment of adult rats is also weakened by the timing of changes in its activity. When measured directly, as opposed to inferring its activity from measuring total SOD activity (4, 13), Cu,ZnSOD activity is not elevated at 60 h of exposure in endotoxin-treated rats; it is elevated in endotoxin-treated rats at 72 h of hyperoxia but by then most of the damage and deaths have occurred in untreated rats (Table V and reference 52).

Table IX. AOE mRNA Half-Life

Conditions	Time	Half-life		
		MnSOD	CAT	GP
		h		
1. Air-saline	48	8.2±0.8 (5)	7.9±0.5 (3)	8.0±0.6 (3)
2. Air-endotoxin	48	7.8±0.5 (3)	8.6±1.3 (3)	6.7±0.5 (3)
3. O_2-saline	48	19.0±2.8 (3)	11.5±1.4 (3)	8.5±0.1 (3)
4. O_2-endotoxin	48	15.4±1.2 (3)	23.2±2.0 (3)	30.9±2.0 (3)
P values	1 vs. 2	NS	NS	NS
	1 vs. 3	< 0.05	NS	NS
	1 vs. 4	< 0.05	< 0.05	< 0.05
	2 vs. 3	< 0.05	NS	NS
	2 vs. 4	< 0.05	< 0.05	< 0.05
	3 vs. 4	NS	< 0.05	< 0.05

Rats were given saline or endotoxin and exposed as described in the legend to Table V. Mean±SE are given. Values in parentheses indicate the number of rats.

Finally, we acknowledge and think it is worth recalling that our assays of AOE activity were made on whole lung with its various cell types. It is therefore distinctly possible that in certain cells AOE activity changed in a manner different from, and undetected by, our analysis of the whole lung. If these undetected responses protected strategically located cells, and thereby prevented lung edema in O_2-endotoxin rats, these changes could be responsible for endotoxin-induced tolerance to O_2. Unraveling this important issue will require detailed morphological studies using in situ hybridization of AOE mRNAs and immunolocalization of AOEs.

Oxidant-induced damage to enzymes as a molecular basis of oxygen toxicity. The notion and evidence that oxygen toxicity is mediated by inactivation of enzymes important to key

Table X. DTT's Effect on Cu,ZnSOD and MnSOD Activity in Lung Extracts from Air-exposed and O_2-exposed Rats

DTT	Enzyme activity			
	Cu,ZnSOD		MnSOD	
	Air-exposed	O_2-exposed	Air-exposed	O_2-exposed
mM	*units · mg⁻¹ protein*			
0	15.9±0.9 (3)	11.1±0.6 (3)	1.2±0.3 (6)	0.18±0.06 (6)
1.5	16.5±1.5 (3)	12.3±0.9 (3)	1.5±0.3 (6)	0.36±0.03 (6)
2.5	ND	ND	1.5±0.3 (3)	0.90±0.06 (3)

Rats were exposed in chambers to air or to > 95% O_2 for 48 h. The lungs were perfused and excised as described in Methods including incubation of the 27,000-*g* supernatant fraction with or without DTT for 15 min at 12°C. The extracts were dialyzed and than assayed for Cu,ZnSOD and MnSOD activity. Mean±SE are given. Figures in parentheses indicate the number of separate experiments each with extracts from different rats. $P > 0.05$ between the means at different concentrations of DTT for each enzyme from air-exposed rats and for Cu,ZnSOD in extracts from O_2-exposed rats; $P < 0.025$ between each concentration of DTT for MnSOD in extracts from O_2 rats. ND, not done.

metabolic pathways are not new (53, 54). However, recent work strengthens the idea that oxidative damage to mitochondrial enzymes plays a key role in oxygen toxicity. This evidence includes the demonstration that (*a*) exposure of Chinese hamster ovary cells to hyperoxia results in inactivation of three key mitochondrial enzymes (NADH dehydrogenase, succinate dehydrogenase, and α-ketoglutarate), an 80% inhibition of oxygen consumption, a 2.5-fold decrease in cellular ATP, and cell death (26); (*b*) pulmonary macrophages from rabbits exposed to hyperoxia exhibit diminished oxidative metabolism and a fall in ATP concentration (55); and (*c*) oxygen consumption is diminished in lung slices from rats exposed to > 95% O_2 (56) and in mitochondria from O_2-exposed rats (57). To these observations we add the suggestion that the increased production of superoxide during hyperoxia results in a fall in MnSOD activity that in turn worsens the effect of the enhanced formation of superoxide and thereby in a major way contributes to the loss of mitochondrial function during hyperoxia.

Our observation of the fall in lung activity of MnSOD during 48 h of hyperoxia, and of the induction of partial recovery by in vitro addition of the reducing agent DTT to the extracts from these lungs, indicates the fall in MnSOD activity represents, at least in part, oxidation of thiol moieties of the enzyme. In that MnSOD contains two cysteine residues, one of which is in its leader sequence (58), it is possible oxidation of the latter could impair transport of the enzyme to, and its entry into, mitochondria where it loses its leader sequence and becomes active. Our data do not shed any light on this possibility. A second possibility is that MnSOD activity is diminished, at least in part, by oxidation of the cysteine not on the leader sequence. Our data showing the in vitro recovery of MnSOD by the addition of DTT to a 27,000-*g* lung extract, which by virtue of being subjected to this centrifugal force should be free of mitochondria, indicates at least part of the O_2-induced loss of MnSOD activity is due to oxidation of thiol residues not in the leader portion of the enzyme.

The O_2-induced decrease in MnSOD activity could also be due to a fall in its rate of synthesis in spite of the elevated concentration of MnSOD mRNA. This possibility is supported by the report that there is less MnSOD protein in lungs of rats exposed to hyperoxia than in air-breathing rats (59). However, in that work the amount of enzyme was assayed by immunoblotting equal amounts of protein from lungs of rats exposed to hyperoxia for 50 h compared to air-breathing rats. Because by 50 h of exposure there is substantial lung edema (reference 4 and Tables II and IV), it is likely lung protein was diluted by serum protein and hence that less lung protein was analyzed in O_2-exposed than in air-exposed rats. Nevertheless, decreased synthesis of MnSOD could contribute to its fall in activity in O_2-exposed rats. Finally, especially if the enzyme is oxidatively or otherwise modified during exposure to hyperoxia, increased degradation could contribute to the decline of its activity (60).

Regulation of AOE gene expression. Our results demonstrate that the regulation of AOE gene expression in adult rat lung in response to oxygen, endotoxin, and oxygen plus endotoxin differs between treatments and among the enzymes. The observation that in air-endotoxin rats the concentration of MnSOD mRNA is elevated without an increase in its stability indicates the increase is due to a faster rate of transcription. Regulation of MnSOD expression by this mechanism in response to endotoxin has been suggested from studies using inhibition of RNA synthesis (61–64). A similar interpretation applies to the elevated concentration of GP mRNA in air-endotoxin rats. The prolonged half-life of MnSOD mRNA present in O_2-saline rats indicates at least part of the elevated concentration of MnSOD mRNA under this condition is due to an increase in its stability. The only response that was uniform among MnSOD, CAT, and GP was to the combination of O_2 plus endotoxin; in all cases there was an elevation of the concentration of the enzymes' mRNA due at least in part to a two- to fourfold increase in stability of the mRNAs.

Unlike adult rats, neonatal rats exposed to > 95% O_2 exhibit a uniform AOE response; there is elevation of lung activity of Cu,ZnSOD, MnSOD, CAT, and GP and enhanced stability of the enzymes' respective mRNA (33, 65); in the case of CAT there was no increase in the rate of CAT mRNA synthesis in the lungs of neonatal rats exposed to > 95% O_2 (33), but this was not tested for the other AOEs. This neonatal pattern of O_2-induced increase in mRNA stability in response to hyperoxia is retained in adults for MnSOD but not for CAT or GP; we have not tested the effect of hyperoxia on the stability of Cu,ZnSOD mRNA in adult rats. By contrast, with respect to the stability of MnSOD mRNA, CAT mRNA, and GP mRNA (not tested for Cu,ZnSOD mRNA) in adult rats, the combination of O_2 plus endotoxin results in the same pattern of response as in neonatal lungs from O_2-exposed rats not treated with endotoxin. Put differently, endotoxin treatment induces adult rats to mimic the response of neonatal rats to hyperoxia. The use of enhanced mRNA stability to augment gene expression, although not as common as transcriptional mediated increases in gene expression, is energetically favorable and just as effective as the use of an increased rate of transcription for that purpose (66).

The mechanism by which hyperoxia alone, or in combination with endotoxin, enhanced the stability of AOE mRNAs is unknown. However, an especially interesting possibility, for which there is some support, is that the change in stability is due to a mRNA binding protein whose conformation, synthesis, or degradation is altered during an oxidant stress. A protein(s), which binds to CAT mRNA in a redox-sensitive manner, has been found in extracts of neonatal lung (67). Neonatal rats exposed to hyperoxia have a larger proportion of CAT mRNA binding protein in oxidized form than lungs from air-breathing neonatal rats. There is thus the very interesting possibility that a protein(s), perhaps a metalloprotein, senses the redox state of the cell and, dependent upon the state, is released from, or remains bound to, the AOE's mRNA thereby influencing the RNA's stability and secondarily the synthesis of the enzyme.

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