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Research Article

Pertussis Toxin Treatment Alters Manganese Superoxide Dismutase Activity in Lung

Evidence for Lung Oxygen Toxicity in Air-breathing Rats

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Abstract

Exposure of rats to hyperoxia or to treatment with endotoxin, increases lung manganese superoxide dismutase (MnSOD) gene expression. However, the paths by which these environmental signals are transduced into enhanced MnSOD gene expression are unknown. We now provide evidence that heterotrimeric G proteins are involved in the hyperoxia-induced increase in lung MnSOD gene expression but that pertussis toxin-sensitive G proteins are not involved in the endotoxin-induced elevation of lung MnSOD gene expression. We also show that treating rats with pertussis toxin decreased lung MnSOD activity ~ 50%. This decline in MnSOD activity occurred without a change in the lung activity of copper-zinc SOD, catalase, or glutathione peroxidase. In air-breathing rats, the pertussis toxin-induced decrease in MnSOD activity was associated with the development of lung edema, pleural effusion with a high concentration of protein, and biochemical evidence of lung oxygen toxicity. Compared to air-breathing rats, maintenance of pertussis toxin-treated rats under hypoxic or hyperoxic conditions respectively decreased or increased intrathoracic fluid. Endotoxin treatment elevated lung MnSOD activity and protected pertussis toxin-treated rats from an increase in intrathoracic fluid. (*J. Clin. Invest.* 1994. 93:2482–2489.) Key words: G proteins • catalase • glutathione peroxidase • signal transduction • endotoxins

Introduction

Superoxide, hydrogen peroxide, and the hydroxyl radical are toxic moieties formed under normoxic and hyperoxic conditions by the incomplete reduction of oxygen. Their increased cellular formation during hyperoxia (1) and the harmful effect these molecules have on cellular constituents form the basis for the generally accepted notion that oxygen radicals and hydrogen peroxide are responsible for the cellular toxicity of oxygen. Antioxidant enzymes are an important component of cellular defenses against these dangerous molecules under normoxic and hyperoxic conditions (2–8). Manganese superoxide dismutase (MnSOD, Cu-ZnSOD, catalase, and gluthathione peroxidase [GP][1]) are antioxidant enzymes widely distributed among organisms that metabolize oxygen (4). The SODs catalyze the dismutation of superoxide to hydrogen peroxide; catalase and GP each convert hydrogen peroxide to water and oxygen, thereby reducing the formation of the even more dangerous hydroxyl radical (4).

Exposure of rats to hyperoxia (8) or treatment of rats with endotoxin (8) or with TNF (9) increases the lung concentration of MnSOD mRNA but the path(s) by which these environmental signals are transduced into enhanced MnSOD gene expression is unknown. In pulmonary epithelial-like (L2) cells activation of heterotrimeric G proteins by non–receptor-mediated mechanisms elevates MnSOD mRNA (10). Furthermore, the induction of increased MnSOD mRNA in L2 cells by TNFα (10), and by purinergic agonists (11), is blocked by pertussis toxin (PTX). PTX, an exotoxin of *Bordatella pertussis,* catalyzes the transfer of ADP-ribose from cytoplasmic NAD to heterotrimeric G proteins; the latter mediate signals from plasma membrane receptors to cellular effector molecules (12). ADP-ribosylation of G proteins prevents their interaction with receptors on plasma membranes, thereby blocking the transduction of environmental signals to cellular effector molecules (see reference 13 for a review). The evidence that G proteins of cells in culture are involved in the regulation of antioxidant enzyme gene expression (10, 11) led us to examine the effect of treating rats with PTX on lung antioxidant enzyme activity and MnSOD mRNA concentration during normoxia and hyperoxia, and after endotoxin treatment.

Methods

Animals. We used 21–35-d-old specific pathogen-free male Sprague-Dawley rats (Taconic Farms, Germantown, NY) that were maintained as previously described (8). All rats, except those used for morphological studies (vide infra), were killed by cutting the great vessels of the abdomen under surgical levels of anesthesia (~ 90 mg · kg^{-1} of pentobarbital sodium). The ratio of lung wet to dry weights was obtained on lungs removed without being perfused. For all other biochemical and molecular assays the lungs were perfused via the pulmonary artery with 30 ml of ice-cold 0.15 M NaCl. The perfused lungs were then excised and immediately processed for the various assays or stored at −70°C for later use.

Treatments and exposures. PTX (List Biological Laboratories, Inc., Campbell, CA) was reconstituted in PBS (25 μg · ml^{-1}) and injected intraperitoneally (5 μg · 100 g^{-1} body weight). Control rats were given an equal volume of PBS intraperitoneally. In some rats lipopolysaccharide (endotoxin) from *Salmonella typhimurium* (Sigma Chemical Co., St. Louis, MO) was injected intraperitoneally (500 μg · kg^{-1} body weight) at a concentration of 100 μg · ml^{-1} PBS. Control rats were

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1. *Abbreviations used in this paper:* GP, glutathione peroxidase; GSSG, glutathione disulfide; PTX, pertussis toxin.

given an equal volume of PBS intraperitoneally. In experiments to determine the effect of oxygen concentration on the lungs response to PTX, we exposed rats to 15% O_2, 20.8% O_2, or >95% O_2 in identical 3.5-ft^3 chambers constructed from clear plastic (8).

ADP ribosylation. We injected rats with PBS or PTX as described above and killed them 24 h later. The lungs were perfused with ice-cold saline, minced, and homogenized in 0.25 M sucrose containing 10 mM Tris, pH 7.4, 1.0 mM EDTA, 0.2 mM PMSF, 1.0 $\mu g \cdot ml^{-1}$ leupeptin, and 0.0002 $U \cdot ml^{-1}$ of aprotinin (medium A). To obtain lung membranes the crude extract was filtered through cheesecloth, the filtrate was diluted to 6% weight/volume by adding medium A and centrifuged at 1,478 *g* for 30 min at 0–4°C (14, 15). The pellet that formed was resuspended in medium A and Percoll was added to a final concentration of 12%. This material was centrifuged at 35,000 *g* for 30 min at 0–4°C and the top band was collected and diluted by adding 4.0 ml of 20 mM Tris, pH 7.4. This material was centrifuged at 9,770 *g* for 45 min at 0–4°C, the sediment was washed with 20 mM Tris, pH 7.4, and recentrifuged at 12,000 *g* for 10 min. The sediment was considered the plasma membrane fraction; this fraction was resuspended in 100 μl of 20 mM Tris, pH 7.4, and the protein concentration was determined (vide infra).

The incubation mixture for the ribosylation reaction included 100 μg of membrane fraction protein, 90 mM Tris, pH 7.4, 50 mM EDTA, 1.0 mM ATP, 10 mM thymidine, 20 mM isoniazid, 100 μM NAD, 5 μl of ^{32}P 6.3 μM NAD, sp act 800 $Ci \cdot mmol^{-1}$, and 10 $\mu g \cdot ml^{-1}$ of preactivated PTX (14). The reaction mixture was incubated for 1 h at 30°C. The reaction was stopped by adding 1.3 ml of 10% trichloroacetic acid. The acid-treated incubation mixture was placed on ice for 10 min and then centrifuged at 12,000 *g* for 5 min, the pellet that formed was dissolved in 60 μl of SDS loading buffer, boiled, and loaded onto a 12% SDS polyacrylamide gel. After electrophoresis the gel was exposed to Hyperfilm (Amersham Corp., Arlington Heights, IL) at −70°C for radioautography.

Assays for enzyme activity. The activity of the SODs, catalase, and GP were determined as previously described (8). To correct for SOD, catalase, and GP activity in lung that was due to blood contamination of lung we sonicated whole blood for 30 s, centrifuged the blood at 27,000 *g* for 20 min, and assayed the 27,000-*g* supernatant fraction for SOD, catalase, and GP activities. The hemoglobin concentration in the lung and in the blood 27,000-*g* supernatant fluids was measured using human hemoglobin (Sigma Chemical Co., St. Louis, MO) as a standard (16). The enzyme activity due to blood contamination was subtracted from the activity measured in lung.

cRNA preparation and solution hybridization for assay of MnSOD mRNA. A rat liver MnSOD cDNA was a gift from Dr. Ye-Shih Ho (Wayne State University, Detroit, MI); an EcoRI-StuI restriction fragment of this cDNA was subcloned into pGEM-3Z vector, and the orientation was determined by sequencing to enable us to transcribe a MnSOD cRNA ^{35}S-labeled antisense probe and a sense-strand standard (17). This probe hybridizes with the six MnSOD mRNA species in rat lung (17). The concentration of MnSOD mRNA was measured in total nucleic acids isolated from the lung (18) using the method of Durnam and Palmiter (19). We added [^3H]actin cRNA (a gift of Dr. Rudolph Warner, University of Miami School of Medicine, Miami, FL) at the start of the process to isolate total nucleic acids to enable us to account for mRNA lost during the extraction process (20).

Chemical assays. In most experiments DNA was assayed by pentose analysis (21); in some experiments it was measured flurometrically with a TKO 100 fluorometer (Hoefer Scientific Instruments, San Francisco, CA); the assay is based on the binding of the fluorescent dye Hoechst 33258 to DNA. Calf thymus DNA served as the standard for both methods. Protein was measured using Coomassie Plus assay reagent from Pierce Chemical Co. (Rockford, IL) with bovine serum albumin as standard.

To measure total glutathione, i.e., GSH and oxidized glutathione (GSSG), 100–500 mg of lung tissue was homogenized as above in 100 mM potassium phosphate buffer, pH 7.5 (22). The homogenate was centrifuged at 27,000 *g* for 45 min at 0–4°C and assayed for total glutathione by observing the change in absorbance at 412 nm after the addition of NADPH. To measure GSSG ~ 500 mg of lung was homogenized, as described above, in 2–3 ml of 100 mM potassium phosphate buffer, pH 7.5, containing 10 mM *N*-ethylmaleimide. The homogenate was centrifuged at 27,000 *g* for 45 min at 0–4°C and the supernatant fluid was assayed for GSSG. A 250-μl sample of the 27,000-*g* supernatant fluid containing 200 μmol exogenous GSSG was passed through a SEP-PAK cartridge (Millipore Corp., Milford, MA); the endogenous GSSG concentration (1–2 μmol) was undetectable compared to the 200 μmol of exogenous GSSG. The percentage of added GSSG recovered from the SEP-PAK was used to correct for losses of endogenous GSSG that occurred when we passed an identical 250-μl sample of the 27,000-*g* supernatant fluid through a SEP-PAK cartridge. The eluate from the SEP-PAK cartridge was assayed for GSSG by observing the change in absorbance at 412 nm after the addition of NADPH. Subtraction of the measured amount of GSSG from the measured amount of total glutathione provided us with the value for GSH.

The carbonyl content of oxidized proteins in lung tissue was measured using 2,4-dinitrophenylhydrazine (23).

Morphological procedures. A bilateral pneumothorax was produced in anesthetized rats by puncturing the diaphragm from its abdominal surface and a thin-walled plastic tube was inserted and tied into the trachea. The rats were killed by infusing cold 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, into the trachea at a transpulmonary pressure of 20 cmH_2O. The trachea was ligated, the lungs were removed from the thorax, and fixation was continued for 2 h at 0–4°C. Blocks prepared from lung tissue were washed three times in fresh 0.1 M sodium cacodylate buffer at 4°C and then postfixed for 1 h in cold 2% osmium tetroxide in 0.1 M sodium cacodylate buffer (pH 7.4). The tissue was dehydrated and embedded in epoxy resin (24) and prepared for ultrastructural analysis as previously described (25).

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 group means was determined by an unpaired *t*-test analysis (26) or by randomized complete-block design (27). The significance of the difference between more than two group means was determined by an analysis of variance (28, 29) or by a randomized complete-block design (27).

Results

ADP ribosylation of components of lung membranes from saline-injected and PTX-treated rats. We incubated lung membranes from saline-treated rats with [^{32}P]NAD in the presence

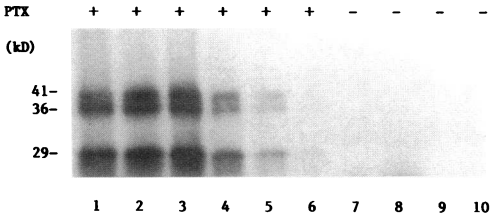

Figure 1. ADP ribosylation of components of lung membranes from saline-injected and from PTX-injected rats. We incubated rat lung membranes with [^{32}P]NAD with or without preactivated PTX (+/−). Lanes *1–3* represent SDS-PAGE and radioautography of membranes from saline-treated rats, lanes *4–6* from PTX-treated rats. Lanes *7–10* are control studies done in the absence of preactivated PTX with membranes from PTX-treated rats (lanes *9* and *10*) or saline-treated rats (lanes *7* and *8*). Molecular mass of the ADP-ribosylated proteins is shown on the left (kD) and was calculated from ^{14}C-labeled standard proteins run on the same gel.

or absence of preactivated PTX. Electrophoresis and radioautography of this material revealed three radioactive bands; their mobility coincided with the mobility of proteins of 41, 36, and 29 kD (Fig. 1). These apparent molecular masses are the same as those reported for proteins ADP-ribosylated as a result of incubating glioma cells with PTX (30, 31). The 41- and the 36-kD material moved as a doublet; this has been previously reported (32) and ascribed to incomplete alkylation of the α subunit of G proteins (33). If treatment of rats with PTX caused in vivo ADP ribosylation from endogenous nonradioactive NAD we would expect little or no ADP ribosylation in vitro from [^{32}P]NAD. This expectation was realized in that radioactivity was absent or markedly diminished in lanes containing material from membranes of PTX-treated rats (Fig. 1). From these results we conclude in vivo PTX treatment resulted in ADP ribosylation of G proteins in the lung.

Treatment of rats with PTX decreases lung MnSOD activity without altering the lung activity of Cu-ZnSOD, catalase, or GP. Treatment of rats with PTX decreased lung MnSOD activity about 50% without a change in the lung activity of Cu-ZnSOD, catalase, or GP (Table I).

MnSOD activity and MnSOD mRNA concentration in lungs of PTX- or endotoxin-treated rats. 36 h after the initial treatments, lung MnSOD activity was 40% less in PTX rats than in saline rats but, at the same time, the concentration of MnSOD mRNA was equal in the two groups (Table II). As previously reported (8), treatment with endotoxin alone elevated lung MnSOD activity and mRNA concentration (Table II). We treated some rats with endotoxin 12 h after the first injection of PTX, a time-point when lung MnSOD activity was already significantly decreased (60% of saline-treated rats [vide infra]); this resulted in MnSOD activity that, 24 h after endotoxin treatment, was 2.6-fold higher in endotoxin-only rats than in saline-treated rats; at the same time MnSOD activity in PTX-endotoxin rats was 3.4-fold higher than in rats given identical PTX treatment without endotoxin (Table II). The magnitude of increase in the lung concentration of MnSOD mRNA was the same in endotoxin-only rats and PTX-endotoxin rats (Table II). It is thus apparent that endotoxin treatment, by an as yet undetermined mechanism, almost completely abrogated the PTX produced fall in MnSOD activity. It is also clear that the endotoxin-induced elevation of MnSOD mRNA was not blocked by treatment with PTX (Table II).

Hyperoxia-induced increase in MnSOD gene expression is diminished by treatment with PTX. Air-PTX rats killed 72 h after the initial injection of PTX had an ~ 50% fall in MnSOD activity but a twofold increase in MnSOD mRNA concentration (Table III). Thus, unlike the lack of change in MnSOD

Table I. Treatment of Rats with PTX Decreases Lung MnSOD Activity without Changing the Lung Activity of Cu, ZnSOD, catalase, or GP

Enzyme activity	Time	Saline	PTX	P
	h	$U \cdot mg^{-1}$ DNA		
MnSOD	24	13.7±1.5 (4)	6.1±0.7 (3)	< 0.05
	72	12.9±1.1 (16)	5.5±0.4 (16)	< 0.001
Cu, ZnSOD	24	102±21 (4)	96±9 (4)	NS
Catalase	24	563±76 (10)	500±59 (10)	NS
GP	24	3.7±0.2 (4)	3.7±0.4 (4)	NS
	72	2.9±0.2 (6)	2.8±0.3 (7)	NS

Mean±SE are given. Figures in parentheses indicate the number of rats. NS = $P > 0.05$. Rats were injected intraperitoneally with PTX (5.0 $\mu g \cdot 100\ g^{-1}$ body weight) or an equal volume of phosphate-buffered 0.15 M NaCl (saline). The time of these injections was designated zero time. Rats to be killed 72 h later were given a second equal dose of PTX or saline 24 h after the initial injection.

Table II. Endotoxin, PTX, and MnSOD Gene Expression

Conditions	MnSOD Activity	MnSOD mRNA
	$U \cdot mg^{-1}$ DNA	(molecules $\cdot mg^{-1}$ DNA) $\times 10^{-6}$
Saline	13.1±1.1 (13)	69±2.0 (4)
PTX	7.7±1.3 (11)	71±5.0 (4)
Endotoxin	34.0±2.8 (11)	319±170 (3)
PTX-endotoxin	26.0±4.1 (7)	385±137 (3)
P values 1 vs. 2	< 0.05	NS
1 vs. 3	< 0.01	< 0.01
1 vs. 4	< 0.01	< 0.01
2 vs. 3	< 0.01	< 0.01
2 vs. 4	< 0.01	< 0.01
3 vs. 4	< 0.01	NS

Mean±SE are given. Figures in parentheses indicate the number of rats. NS = $P > 0.05$. Rats were injected intraperitoneally at zero time as follows: saline rats were given saline; PTX rats and PTX endotoxin rats were given PTX (5.0 $\mu g \cdot 100\ g^{-1}$ body weight); endotoxin rats received saline. 12 h after zero time the rats were injected as follows: saline rats with saline and PTX rats with PTX as at zero time; endotoxin rats received endotoxin (500 $\mu g \cdot kg^{-1}$ i.p.) and PTX-endotoxin rats were given PTX, as at zero time, plus endotoxin (500 $\mu g \cdot kg^{-1}$). All rats were killed 36 h after zero time.

Table III. Hyperoxia, PTX Treatment, and MnSOD Gene Expression

Conditions	MnSOD Activity	MnSOD mRNA
	$U \cdot mg^{-1}$ DNA	(molecules $\cdot mg^{-1}$ DNA) $\times 10^{-6}$
Air-saline	13.0±1.4 (16)	21.0±2.0 (8)
Air-PTX	6.0±0.5 (16)	40.0±5.0 (8)
O_2-saline	21.4±1.9 (17)	184.0±20.0 (8)
O_2-PTX	5.7±0.8 (14)	127.0±13.0 (8)
P values 1 vs. 2	< 0.01	< 0.01
1 vs. 3	< 0.01	< 0.001
1 vs. 4	< 0.01	< 0.001
2 vs. 3	< 0.01	< 0.001
2 vs. 4	NS	< 0.001
3 vs. 4	< 0.01	< 0.001

Mean±SE are given. Figures in parentheses indicate the number of rats. NS = $P > 0.05$. Rats were injected intraperitoneally with PTX (5.0 $\mu g \cdot 100\ g^{-1}$ body weight) or an equal volume of saline immediately before and 24 h after the onset of exposure. Rats were placed in identical chambers and exposed to air or to > 95% O_2 for 72 h.

Table IV. The Onset of PTX-induced Fall in Lung MnSOD Activity Precedes the Onset of Increase in the Ratio of Lung Wet Weight to Dry Weight

Time	MnSOD			Wet weight · dry weight^{-1}		
	Saline	PTX	%‡	Saline	PTX	%‡
h	*U · mg DNA^{-1}*					
3	12.9±1.1 (6)	11.7±0.8 (6)	91	5.16±0.03 (6)	5.10±0.06 (5)	98.8
6	12.9±3.0 (4)	10.3±2.6 (4)	80	5.25±0.06 (4)	5.25±0.05 (4)	100.0
12	13.0±1.0 (5)	8.9±0.9 (5)*	68	5.16±0.03 (5)	5.78±0.2 (4)*	112.0
24	13.7±1.5 (4)	6.1±0.7 (3)*	45	ND		
48	12.9±0.8 (4)	6.2±1.0 (3)*	48	5.39±0.06 (5)	5.98±0.08 (5)*	110.9
72	12.9±1.1 (16)	5.5±0.4 (16)*	48	5.10±0.1 (5)	5.90±0.01 (5)*	115.6

Mean±SE are given. Figures in parentheses indicate the number of rats. * $P < 0.05$ between saline and PTX group. ND, not determined. ‡ PTX value as a percentage of saline value. Rats were injected intraperitoneally with PTX (5.0 μg · 100 g^{-1} body weight) or an equal volume of saline at zero time. Rats killed 48 or 72 h later were given a second equal dose of saline or PTX 24 h after zero time.

mRNA concentration 36 h after the onset of treatment with PTX (Table II), after an additional 36 h the lung concentration of MnSOD mRNA increased twofold (Table III). This rise in mRNA concentration in air-PTX rats did not result in an elevation of MnSOD activity (Table III).

O_2-saline rats, as previously reported (8), had elevated lung MnSOD activity and mRNA concentration (Table III). By contrast, O_2-PTX rats did not have an increase in lung MnSOD activity; rather, the activity in O_2-PTX rats was depressed to the same extent as in air-PTX rats (Table III). Treatment with pertussis toxin blunted by ~ 60% the O_2-induced elevation of lung MnSOD mRNA concentration: hyperoxia resulted in an 8.8-fold rise in MnSOD mRNA concentration in saline rats but only a 3.2-fold increase in PTX-rats (Table III).

Lung edema and pleural effusion in PTX-treated air-breathing rats. Very early studies have shown lung edema and pleural effusion occur in air-breathing mice treated with PTX but the basis for their occurrence has remained unclear (34). Oxidant stress also produces lung edema and pleural effusion (6). Because it is possible the low MnSOD activity in air-PTX rats resulted in an oxidant stress even in air-breathing rats we compared the time course of the fall in MnSOD activity and of the development of lung edema as indicated by an increase in the ratio of the lung's wet weight to dry weight. Although the intergroup values for MnSOD activity did not attain statistical significance until 12 h after treatment it seems apparent from the time course values that a fall in MnSOD activity had begun before lung edema was detected (Table IV). The pleural effusions in PTX-treated rats had a high concentration of protein; it was 55±1.0% (mean±SE, $n = 3$) of the protein concentration in serum 24 h after a single injection of PTX. 72 h after the first injection of PTX (a second injection was given 24 h after the first) the protein concentration in the pleural fluid was 54±0.9% ($n = 5$) of the protein concentration in serum.

Lung glutathione and oxidized glutathione and the carbonyl content of lung proteins in rats treated with PTX. The time course of the decline in MnSOD activity and of the appearance of lung edema indicates the fall in activity preceded the appearance of edema (Table IV). This is consistent with the notion that oxidative stress led to lung damage that resulted in edema. We therefore tested for the presence of biochemical changes compatible with the occurrence of oxidative stress. Treatment with PTX decreased the lung concentration of reduced glutathione ~ 35% but did not alter the concentration of oxidized glutathione (Table V). The carbonyl content of lung proteins, an index of the oxidative modification of protein (23), was about twofold higher in rats treated with PTX than in saline-treated rats (Table V).

Exposure of PTX-treated rats to a low (15%) or a high (95%) concentration of oxygen. To further pursue the possibility that PTX treatment resulted in an oxidant stress to the lung we took advantage of two observations about the generation by cells of the toxic oxygen metabolites superoxide and hydrogen peroxide: a low oxygen tension decreases their production (35, 36) and a high oxygen tension increases their production (1). We therefore asked if the magnitude of lung edema would be diminished in rats treated with PTX and placed in 15% O_2. We found that at 24 and 48 h the extent of lung edema was significantly less in 15% O_2-PTX rats than in air-PTX rats (Table VI). Exposure to 15% O_2 did not affect the fall in MnSOD activity produced by treatment with PTX (Table VI). We next sought to determine if hyperoxia would worsen the evidence of capillary leak. Exposure to > 95% O_2 increased the lung's wet weight-to-dry weight ratio equally in air-PTX and O_2-PTX rats (Table VII). However the volume of pleural fluid was sixfold greater in O_2-PTX than in air-PTX rats (Table VII).

Table V. Lung Glutathione and the Carbonyl Content of Proteins in the Lung of Rats Treated with PTX

Conditions	GSH	GSSG	Carbonyl content
	nmol · mg^{-1} DNA		
Saline	137±8.4 (9)	1.0±0.1 (9)	1280±64 (4)
PTX	88±4.9 (12)	1.0±0.1 (12)	2761±612 (4)
P values	< 0.01	NS	< 0.05

Mean±SE are given. Figures in parentheses indicate the number of rats. NS = $P > 0.05$. Rats were injected intraperitoneally with PTX (5.0 μg · 100 g^{-1} body weight) or with an equal volume of saline at zero time and 12 or 24 h later. Measurements of GSH and GSSG were made on rats killed 72 h after zero time injection; measurements of carbonyl content were made on rats killed 36 h after the zero time injection.

Table VI. Lung Edema in PTX-treated Rats Exposed to 15% O_2

Conditions	24 h	48 h	MnSOD
	wet weight · dry weight^{-1}		*U · mg^{-1} DNA*
Air-saline	5.0±0.0 (4)	5.1±0.1 (8)	12.0±1.6 (4)
Air-PTX	6.1±0.1 (4)	5.7±0.1 (7)	6.6±1.2 (4)
15% O_2-saline	5.1±0.0 (4)	5.2±0.0 (7)	16.5±1.6 (4)
15% O_2-PTX	5.5±0.2 (4)	5.5±0.1 (7)	7.8±0.6 (4)
P values 1 vs. 2	< 0.01	< 0.01	< 0.01
1 vs. 3	NS	NS	NS
1 vs. 4	< 0.01	< 0.01	< 0.05
2 vs. 3	< 0.01	< 0.01	< 0.01
2 vs. 4	< 0.01	< 0.01	NS
3 vs. 4	< 0.01	< 0.01	< 0.01

Mean±SE are given. Figures in parentheses indicate the number of rats. NS = $P > 0.05$. Rats were injected intraperitoneally with PTX (5.0 μg · 100 g^{-1} body weight) or with an equal volume of saline immediately before and 24 h (48 h exposed rats only) after the onset of exposure. Rats were exposed in identical chambers to 20.8% O_2 (air) or to 15% O_2 for 24 or 48 h. The values for MnSOD activity represent rats killed 48 h after the onset of exposure.

Lung edema and pleural fluid in rats treated with PTX and endotoxin. Treatment with endotoxin increases lung MnSOD activity (reference 8 and Table II) and protects rats against the potential damaging effect of hyperoxia (6, 8). Since we suspected the PTX-induced fall in MnSOD activity results in oxidative stress and damage to the lung even in air-breathing rats, we examined the effect of endotoxin treatment on the extent of lung edema and the magnitude of pleural effusion in rats treated with PTX. Compared to their respective control group, rats treated with PTX plus endotoxin increased their lung wet weight-to-dry weight ratio to the same degree as rats treated with only PTX (Table VIII). However, treatment with endotoxin almost completely eliminated the development of pleural effusion in PTX-treated rats (Table VIII).

Table VII. Lung Edema and Pleural Fluid in PTX-treated Rats Exposed to > 95% O_2

Conditions		Pleural fluid
	wet weight · dry weight^{-1}	*ml*
Air-saline	5.1±0.1 (13)	0.0±0.0 (32)
Air-PTX	5.6±0.0 (5)	0.3±0.1 (32)
O_2-saline	5.9±0.0 (5)	0.0±0.0 (21)
O_2-PTX	5.6±0.1 (5)	2.1±0.2 (18)
P values 1 vs. 2	< 0.01	< 0.01
1 vs. 3	< 0.01	NS
1 vs. 4	< 0.01	< 0.01
2 vs. 3	< 0.01	< 0.01
2 vs. 4	NS	< 0.01
3 vs. 4	< 0.01	< 0.01

Mean±SE are given. Figures in parentheses indicate the number of rats. NS = $P > 0.05$. Rats were injected intraperitoneally with PTX (5.0 μg · 100 g^{-1} body weight) or an equal volume of saline immediately before and 24 h after the onset of exposure. Rats were placed in identical chambers and exposed to air or to > 95% O_2 for 72 h.

Table VIII. Effect of Endotoxin Treatment on PTX-induced Lung Water and Pleural Fluid

Conditions		Pleural fluid
	wet weight · dry weight^{-1}	*ml*
Saline	5.5±0.1 (10)	0.0±0.0 (19)
PTX	6.3±0.1 (11)	2.0±0.2 (20)
Endotoxin	5.2±0.1 (9)	0.0±0.0 (19)
PTX → endotoxin	5.9±0.1 (9)	0.2±0.1 (16)
P values 1 vs. 2	< 0.01	< 0.01
1 vs. 3	NS	NS
1 vs. 4	< 0.01	NS
2 vs. 3	< 0.01	< 0.01
2 vs. 4	< 0.01	< 0.01
3 vs. 4	< 0.01	NS

Mean±SE are given. Figures in parentheses indicate the number of rats. NS = $P > 0.05$. Rats were injected intraperitoneally at zero time as follows: saline rats with saline; PTX and PTX-endotoxin rats with PTX, 5 μg · 100 g^{-1} body weight. 12 h after the zero time injection the rats were injected as follows: saline rats with saline and PTX rats with PTX as at zero time; endotoxin rats received endotoxin, 500 μg · kg^{-1} i.p.; PTX-endotoxin rats were given PTX as at zero time plus endotoxin (500 μg · kg^{-1}). All rats were killed 36 h after the first injection.

Morphology. Rats treated with PTX and allowed to remain in air developed morphological evidence of edema in the lung's gas-exchange region as indicated by the marked widening of the interstitial space; similar enlargement was present in O_2-saline rats (Fig. 2). In addition to marked widening of the interstitial space, O_2-PTX rats exhibited the presence of a large amount of intraalveolar floccular material (Fig. 2).

Discussion

Signal transduction in the regulation of antioxidant enzyme gene expression. The antioxidant enzymes MnSOD, Cu-ZnSOD, catalase, and GP are encoded on nuclear genes and exhibit some degree of specificity in their subcellular sites of action (37–39). Superoxide and hydrogen peroxide, substrates for these enzymes, are each produced at several subcellular sites and the rate of production of each probably varies among the sites (40). It therefore seems mandatory that cells have signaling mechanisms whose activation results in changes in antioxidant enzyme gene expression appropriate to meet site specific needs for scavenging superoxide and hydrogen peroxide. Heterotrimeric G proteins transduce signals that activate many cellular processes including some, such as ion transport, that have a major effect on total cellular oxygen consumption and, presumably, on the production of superoxide and hydrogen peroxide. G proteins may also be involved in intracellular trafficking of proteins (41) such as may be required to direct antioxidant enzymes to their appropriate subcellular organelles and sites of action. These considerations suggested to us that heterotrimeric G proteins might serve in transduction systems that link environmental signals, processes that influence cellu-

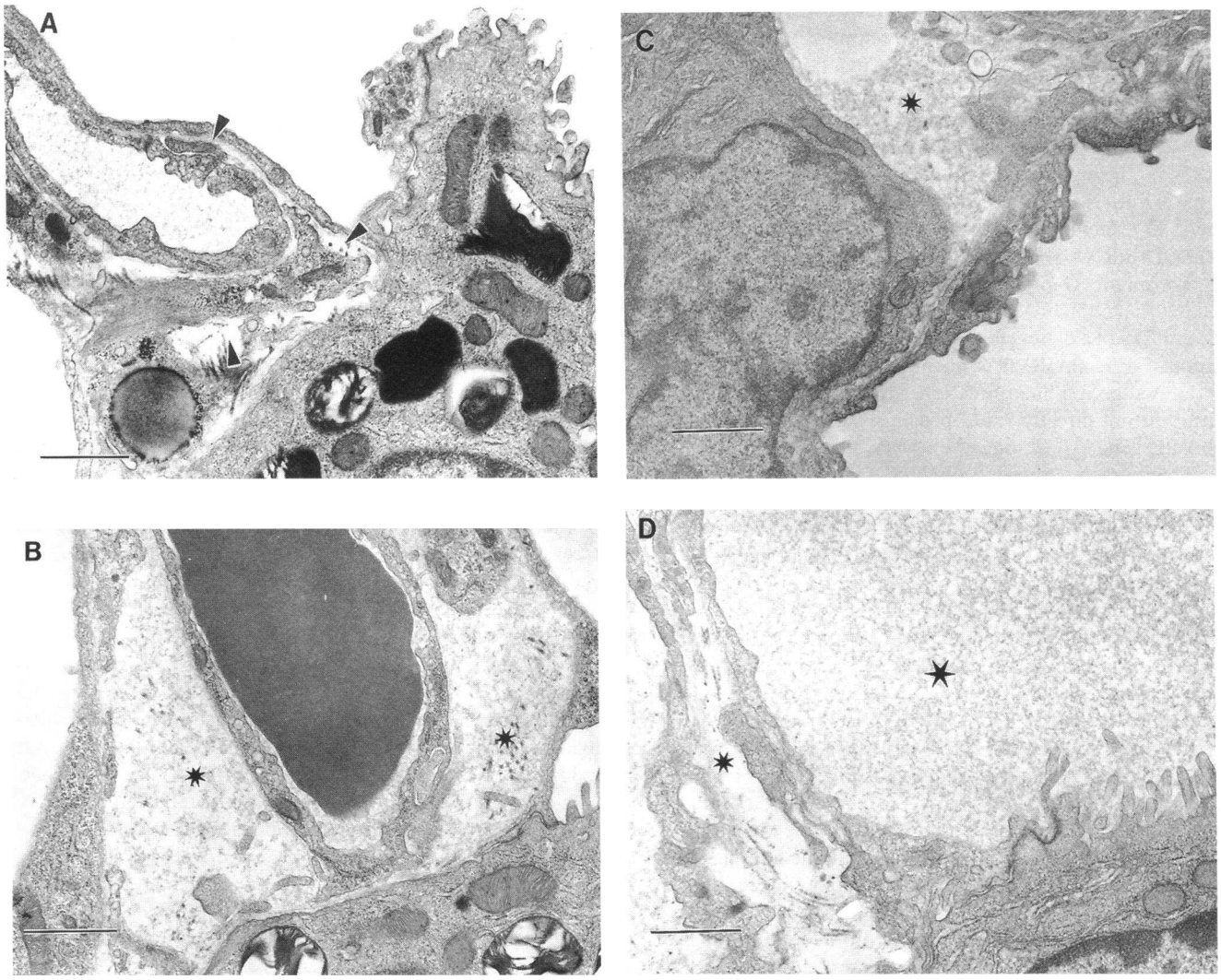

Figure 2. Electron microscopic evidence of edema in the alveolar-capillary region. Rats were injected intraperitoneally with pertussis toxin (5.0 $\mu g \cdot 100\ g^{-1}$ body weight) or an equal volume of saline before and 24 h after the onset of exposure. Rats were exposed to air or > 95% O_2 for 72 h. (*A*) Saline-treated, air-exposed. (*B*) PTX-treated, air-exposed. (*C*) Saline-treated O_2-exposed. (*D*) PTX-treated O_2-exposed. Arrowhead indicates normal interstitial space in the alveolar-capillary region. Small star indicates greatly widened interstitial space filled with floccular material. Large star depicts alveolar space filled with floccular material. Scale markers, 1 μm.

lar metabolic rate, and mechanisms that modulate antioxidant enzyme gene expression.

Prior work provided the following evidence to support the notion that heterotrimeric G proteins are involved in the regulation of antioxidant enzyme gene expression in pulmonary epithelial-like (L2) cells: (*a*) activation of heterotrimeric G proteins with aluminum fluoride or with a nonhydrolyzable analogue of guanosine triphosphate results in elevation of the mRNAs of MnSOD, catalase, and GP (10); (*b*) PTX, which inactivates a subfamily of heterotrimeric G proteins (13), blocks the transcriptionally regulated (42), receptor activated (43), increase in MnSOD mRNA concentration induced by TNF-α as well as the transcriptionally regulated increase in MnSOD mRNA produced by aluminum fluoride (10); and (*c*) purinergic agonists elevate the concentration of MnSOD mRNA in L2 cells and this is also prevented by PTX (10).

Our present work provides evidence heterotrimeric G proteins mediate the regulation of MnSOD gene expression in rat lung. For example, O_2-saline rats exhibited an 8.8-fold rise in MnSOD mRNA concentration above the concentration in air-saline rats (Table III); by comparison, O_2-PTX rats demonstrated a 3.2-fold rise in MnSOD mRNA concentration above the concentration in air-PTX rats (Table III), a fold increase that is 64% lower than in O_2-saline rats. These observations raise the important possibility of the presence of a O_2-responsive membrane protein, perhaps a redox-sensitive metalloprotein, that serves as an O_2 receptor. The latter, through G proteins, could mediate changes in MnSOD gene expression in response to changes of environmental O_2 concentration.

The blunted response was not the consequence of an irrevocable inability of the lung to increase MnSOD mRNA because PTX rats did elevate their lung MnSOD mRNA after treatment with endotoxin (Table II). In the same vein, the low MnSOD activity in PTX rats is not due to an irrevocable inability of lungs of PTX rats to increase MnSOD activity. This is shown by the increase in lung MnSOD activity induced by

endotoxin in rats pretreated with PTX (Table II). This latter response to endotoxin is particularly impressive because endotoxin was administered 12 h after the first dose of PTX (Table II), a time when PTX-only rats already had a 32% decrease in MnSOD activity (Table IV). It is therefore clear that lungs of PTX-rats are capable of elevating MnSOD activity and mRNA. These data also indicate the endotoxin induction of MnSOD mRNA is not mediated via PTX-sensitive heterotrimeric G proteins but that the hyperoxia-induced increase in MnSOD mRNA is partially mediated through PTX-sensitive heterotrimeric G proteins. We recognize the possibility the blunted elevation of MnSOD mRNA in O_2-PTX rats may be a secondary not a direct effect of heterotrimeric G protein inactivation. For example, it is possible the action of PTX on G proteins alters a cellular function, such as ion transport, that in turn, but not directly via G proteins, signals an alteration in MnSOD gene expression.

The marked depression of MnSOD activity in rats treated with PTX is present before there is a change in the concentration of MnSOD mRNA (Table II). The low activity could reflect a decreased rate of MnSOD synthesis, an increased rate of degradation, inactivation of the enzyme, a failure of activation of the enzyme, or a combination of these events. Failure of activation could be due to a defect in the transport or entry of the protein into mitochondria where it is activated. Whatever the mechanism for the low MnSOD activity in air-PTX rats, its occurrence suggests PTX-sensitive G proteins modulate the steady-state level of MnSOD activity.

PTX, MnSOD activity, and oxidative stress. Treatment with PTX has been long known to cause lung edema and pleural effusion but the basis for this effect of the toxin has been unknown (34). Two observations indicate the findings in air-PTX rats represent increased alveolar-capillary leak; first, the pleural fluid is rich in protein and the floccular nature of the material in the greatly widened interstitium (Fig. 2 *B*) suggests the excess fluid in that location is also protein-rich; the latter finding is also present in PTX-rats exposed to hyperoxia (Fig. 2 *D*).

We suggest the increased alveolar-capillary permeability in air-PTX rats reflects the presence of oxygen toxicity under normoxic conditions. The following observations support this notion: (*a*) the low MnSOD activity in air-PTX rats, unless accompanied by decreased production of superoxide in mitochondria, and perhaps in the cytosol, would shift the oxidant-antioxidant balance in favor of oxidation. Treatment with endotoxin substantially increased MnSOD activity and also markedly decreased the volume of intrathoracic water (Table VIII). (*b*) A hypoxic environment, which decreases the production of superoxide (35, 36), resulted at 24 h of exposure in a 7.8% increase in lung wet-to-dry weight ratio compared to a 22% increase in air-PTX rats and, at 48 h, a 5.7% increase in the wet-to-dry weight ratio compared to a 12% elevation in air-PTX rats (Table VI). (*c*) Hyperoxia, which increases the production of superoxide and hydrogen peroxide (1), resulted in a marked increase in pleural fluid in O_2-PTX rats compared to air-PTX rats (Table VII). (*d*) The lung concentration of GSH, which may be substantially diminished under conditions of oxidant stress with or without an elevation of GSSG (44), was 35% lower in air-PTX rats than in air-saline rats (Table V). (*e*) The introduction of carbonyl groups into amino acids of proteins is a hallmark of the oxidative modification of proteins (23). We thus consider the twofold higher carbonyl content of proteins in PTX rats compared to saline rats as the most direct evidence that treatment with PTX led to oxidative damage in air-breathing rats (Table V).

There is now rather strong evidence that oxidative damage to mitochondrial enzymes plays a key role in the development of oxygen toxicity and that MnSOD, perhaps because of its intramitochondrial location, is especially important as an antioxidant enzyme. Evidence supporting the role of oxidative damage of mitochondrial enzymes in the development of oxygen toxicity includes the following: (*a*) exposure of cells to hyperoxia leads to inactivation of three important mitochondrial enzymes (NADH dehydrogenase, succinic dehydrogenase, and α-ketoglutarate), an 80% decline in oxygen consumption, a 2.5-fold decrease in cellular ATP concentration, and cell death (45); (*b*) macrophages from lungs of rabbits exposed to hyperoxia exhibit diminished oxidative metabolism and a fall in ATP concentration (46); and (*c*) oxygen consumption is decreased in lung slices from rats exposed to $> 95\%$ O_2 (47) and in mitochondria from O_2-exposed rats (48). The gradual rise in MnSOD mRNA over 72 h in air-PTX rats may reflect a signal generated by the production of more superoxide in mitochondria than could be adequately dismutated because of the low MnSOD activity. Evidence that among the antioxidant enzymes MnSOD is especially important in lung tolerance to hyperoxia includes the report that transgenic mice with high lung MnSOD activity tolerate hyperoxia better than normal mice (49), and, the finding that a rise in lung MnSOD activity in adult rats during exposure to hyperoxia distinguishes O_2-tolerant from nontolerant rats (8).

If low MnSOD activity is the basis for oxygen toxicity in air-breathing rats treated with PTX, it would explicate the long unknown cause of PTX induced lung edema and pleural effusion (34). Because the toxicity of irradiation is believed to be mediated by oxyradicals, the PTX-induced fall in MnSOD activity may also account for the early recognized increased susceptibility of PTX treated mice to irradiation (50). The effect of PTX on MnSOD activity should be considered in experiments using the toxin to analyze G protein function. Finally, in view of the observations that MnSOD protects cells against damage due to irradiation (51) the possibility that the local administration of PTX to tumors might make them more radiosensitive should be considered.

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