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Hyperoxia Increases Oxygen Radical Production in Rat Lungs and Lung Mitochondria*

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An increased production of oxygen radicals has been postulated to be a major factor in the etiology of lung damage during hyperoxia. Mitochondrial electron transport was inhibited with CN^- or with antimycin A in both rat lung slices and isolated mitochondria. CN^-- or antimycin A-insensitive O_2 uptake was measured polarographically, as a function of P_{O_2}, and served as an approximate index of intracellular O_2^- and H_2O_2 production. In lung slices, CN^--resistant respiration increased as a function of P_{O_2}, accounting for 9% of total respiration in air and becoming 18% of total respiration when the tissue was incubated in 85% O_2. CN^--resistant respiration in isolated mitochondria also increased as a function of P_{O_2} rising from 0 at 15% O_2 to 1.34 nmol of O_2 consumed/min•mg of mitochondrial protein at 85% O_2. Mitochondria accounted for 15 ± 3% of the CN^--resistant respiration in rat lungs under hyperoxic conditions and released H_2O_2 extramitochondrially at a rate of 50 nmol/min/1.5 g of rat lung. The H_2O_2 generation is dependent on P_{O_2} and substrate and most, if not all, H_2O_2 arises from dismutation of O_2^- produced by autooxidation of respiratory chain components. 2,4-Dinitrophenol increased respiratory chain O_2^- production in a dose-dependent fashion. This phenomenon occurred when mitochondria were treated with nitroaromatic compounds which can be reduced to nitroanion free radicals capable of reducing O_2 to O_2^-. Nonreducible uncouplers such as salicylate did not increase mitochondrial O_2^- generation, suggesting that uncoupling, per se, does not necessarily favor increased rates of mitochondrial O_2^- production. These data suggest that hyperoxia increases the pulmonary production of oxygen radicals and that mitochondria contribute significantly to this phenomenon.

This report describes efforts to characterize the effect of oxygen concentration on the production of partially reduced species of oxygen in rat lungs, and estimates the contribution of lung mitochondria.

Gerschman (1) first proposed that oxygen toxicity may be caused by the formation of free radicals which could then lead to destructive oxidations. She further noted that oxygen at 20% is potentially toxic, and speculated that its gradual accumulation in the atmosphere had provoked the evolution of cellular defenses (1). These suppositions are supported by the observation that anaerobic bacteria, which are deficient in catalase and superoxide dismutase, will not survive under normoxic conditions, and that facultative bacteria are more tolerant of hyperoxia after superoxide dismutase is induced (2). We wished to characterize the effect of oxygen concentration on the production of potentially toxic reduced species of oxygen in the lung, since this is the principal site of injury in mammals breathing increased concentrations of normobaric oxygen. Severe morphological and functional alterations in the lung are caused by inhaling elevated concentrations of oxygen, often resulting in death (3-5). Extensive injury occurs primarily in the lungs since the oxygen transport system largely insulates remote tissues from changes in alveolar P_{O_2}. In contrast, alveolar cells receive oxygen by direct diffusion from the gas phase, so hyperoxia could result in profound alterations in intracellular P_{O_2}.

Enzymatic or spontaneous dismutation of O_2^- yields H_2O_2 + O_2 (6). O_2^- and H_2O_2, the one-electron and two-electron reduced forms of O_2, can react together to form even more deleterious oxygen species such as the hydroxyl radical (OH·) (7-9). Iron compounds have been shown to catalyze the reduction of H_2O_2 by O_2^- (10, 11), according to Equations 1 and 2.

$$O_2^- + Fe^{3+} \rightarrow O_2 + Fe^{2+} \quad (1)$$

$$Fe^{2+} + H_2O_2 \rightarrow OH\cdot + OH^- \quad (2)$$

Considerable data has accumulated suggesting that hyperoxia increases intracellular production of O_2^- and H_2O_2. This suggestion derives from the observation that superoxide dismutase and/or catalase protect eukaryotic cells from oxygen toxicity (12). In *Escherichia coli*, preinduction of superoxide dismutase is critical for resistance to toxicity due to hyperoxia (13, 14); this same phenomenon is observed in mammals. Adult rats exposed to 85% oxygen for 7 days are tolerant to a subsequent prolonged exposure to 100% oxygen, whereas normal adult rats uniformly die within 72 h during exposure to 100% oxygen (15). The development of oxygen tolerance in rats is correlated with an increase in lung superoxide dismutase activity. Importantly, the subsequent decrease of superoxide dismutase activity following transference of 100%-oxygen tolerant rats to room air is correlated with a loss of tolerance to hyperoxia. Additionally, animals such as guinea pigs and mice which do not increase their pulmonary superoxide dismutase activity upon exposure to 85% oxygen will not develop tolerance to 100% oxygen (15).

There are numerous potential sources of O_2^-, yet the relative quantitative contribution of these sources to the O_2^- production of an organ is poorly understood. Cells such as polymorphonuclear leukocytes, macrophages, and platelets secrete O_2^- extracellularly (12). Several mammalian cell cytoplasmic components, including cofactors, enzymes, and organelles, produce substantial amounts of O_2^-. Superoxide dis-

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mutase inhibits the activity of several enzymes, including galactose oxidase (16) and tryptophan dioxygenase (17), emphasizing the role of O_2^- in normal cellular metabolism. The univalent and divalent reduction of oxygen by xanthine oxidase (18) and ferredoxin (19) increases with oxygen concentration. This leads to the expectation that an elevation of P_{O_2} in inspired gases may lead to enhanced pulmonary intracellular O_2^- and H_2O_2 production. Nonenzymatic sources of O_2^- include autooxidizable electron-transferring components, heme-containing proteins, ferredoxins, thiols, and catecholamines (20). These autooxidations should increase as a function of P_{O_2}.

Mitochondria are potential sources of H_2O_2 in cells (21). While the specific site of mitochondrial H_2O_2 production is controversial (22-26), it is agreed that respiratory inhibitors blocking electron flow on the substrate side of the *b*-cytochromes suppress H_2O_2 formation (25), while antimycin A, blocking electron flow on the oxygen side of the *b*-cytochromes, enhances H_2O_2 production (22). Studies with superoxide dismutase free submitochondrial particles, which permit assay of O_2^- production and parallel quantitation of H_2O_2 production (with added superoxide dismutase) have demonstrated that the rate of respiratory chain O_2^- production is almost twice that of H_2O_2 normally produced by the mitochondrial respiratory chain (22, 24). Thus, mitochondrial H_2O_2 is predominantly due to generation of O_2^-, which subsequently undergoes dismutation, according to Equation 3.

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2 \quad (3)$$

We now describe the effect of oxygen concentration on oxygen consumption of rat lung tissue slices and mitochondria in which normal cytochrome-mediated oxygen reduction to H_2O is blocked by CN^-, azide, or antimycin A. This respiratory inhibitor-resistant respiration is an indirect measure of the partial reduction of oxygen to O_2^- and H_2O_2. The contribution of mitochondria to whole lung CN^--resistant respiration and the effect of respiratory chain uncouplers on CN^--resistant respiration is also discussed.

EXPERIMENTAL PROCEDURES

Materials—Cytochrome *c* (type III), xanthine oxidase, fraction V bovine serum albumin, xanthine, D-α-tocopherol acetate (type III), 2,4-dinitrophenol, KCN, antimycin A, rotenone, NADH, NADPH, and ADP were obtained from Sigma. Bovine liver catalase was obtained from Calbiochem-Behring. Nitrofurantoin, salicylic acid, and *o*-nitrobenzoate were from J. T. Baker Chemical Co. The manganese form of superoxide dismutase was purifed from human liver according to McCord *et al.* (28). Oxygen consumption was measured polarographically using a 1.5-ml waterjacketed cell (Gilson Medical Electronics, Middleton, WI) fitted with a Clark oxygen probe (Model 4004, Yellow Springs Instrument Co.).

Animals—Specific pathogen-free male Sprague-Dawley rats weighing 300-350 g were obtained from Charles River Breeding Laboratories, Wilmington, MA. Lungs studied from randomly selected rats had no histological evidence of infection.

Oxygen Exposure—All exposures were continuous and done at 22-24 °C for seven days in polystyrene chambers (37 × 47 × 41 cm) as previously described (15). Oxygen concentration was maintained at 85 ± 2% by mixing pure oxygen with air and maintaining flow rates sufficient to provide eight to nine gas volume changes/h. CO_2 was maintained at less than 0.5% concentration. Control animals were exposed to air at similar flow rates in identical chambers. Rats were provided Purina rat chow and water *ad libitum*.

Tissue Preparation—Rats were killed by cervical dislocation. When mitochondrial preparations were made, lungs were ventilated with a Harvard small animal respirator following tracheostomy, using a maximum positive ventilation pressure of 10 cm of H_2O. In rapid sequence, the inferior vena cava and aorta were transected, the pulmonary artery was cannulated, the left atrium was incised, and the lungs were perfused with 10 ml of ice-cold buffer containing 0.25 M sucrose, 2 mM EDTA, 5 mM Tris-HCl, and 0.5% fraction V bovine serum albumin, pH 7.4. Lung mitochondria were isolated in the same buffer according to the Spear and Lumeng (29) modification of the method of Reiss (30). For lung tissue slice studies, nonperfused lungs were excised from rats following inferior vena cava and aorta transection. Lung tissue slices were made using a McIlwain tissue slicer (Brinkmann) set for 1.0-mm thickness.

Polarographic Measurement—Oxygen consumption was measured in four to five randomly selected, 1-mm-thick lung tissue slice sections which weighed a total of 40 mg. Determinations were made at 30 °C in Krebs-Ringer phosphate buffer, pH 7.4, containing 5 mM glucose, within 1 hour after lung slice preparation.

Mitochondrial oxygen consumption was measured at 30 °C using an incubation medium consisting of 105 mM KCl, 20 mM KH_2PO_4, 0.1 mM EDTA, 0.5% fraction V bovine serum albumin, pH 7.4. Each determination was made using approximately 1 mg of mitochondrial protein. Mitochondrial ADP/O and respiratory control ratios were calculated according to Estabrook (31). Various oxygen tensions were established in solution by bubbling with a gas containing O_2 and N_2 mixed in proper ratios. The oxygen consumption at various oxygen tensions was calculated from the slope of the polarograph output as an actively respiring specimen reached the P_{O_2} of interest. The polarograph cell was equipped with a waterjacket and center-hole stopper for adding substrates and inhibitors. In order to minimize the diffusion of oxygen out of the polarograph cell during measurements made at above-ambient P_{O_2}, the cell was encased with a Lucite box. The box was flushed with the appropriate oxygen concentration, which decreased the oxygen gradient between the stoppered polarograph cell and surrounding gas. Tissue respiration consumed a small per cent of the incubation medium oxygen, so metabolism did not produce further oxygen gradients between the polarograph cell and Lucite box.

Biochemical Analysis—Cytochrome *c* oxidase activity was assayed using 30 μM reduced cytochrome *c* in 50 mM potassium phosphate buffer, pH 7.4. An ϵ_M of cytochrome *c* of 2.1×10^4 (32) was used for all calculations. Rotenone-insensitive NADPH cytochrome *c* reductase activity was assayed according to Sottocasa *et al.* (33). Catalase activity was measured according to Bergmeyer (34). DNA was determined using diphenylamine (35). Protein was quantitated by the method of Lowry *et al.* (36).

Statistical Analysis—Statistical significance reported in Tables II and III represents comparisons between paired samples of treated mitochondria and control mitochondria from the same animal using Student's two-tailed *t* test. $p < 0.025$ was considered to be significant.

RESULTS

The Effect of Hyperoxia on CN^--resistant Respiration in Lung Tissue Slices—Polarographic measurement of lung tissue slice oxygen consumption compares favorably with manometric measurements performed by O'Neil *et al.* (37) using rat lungs of similar size. These investigators reported a calculated oxygen consumption of 1.61 ± 0.13 μmol of O_2/min·rat lung in 1 mm-thick lung slices having an incubation head space gas consisting of 95% O_2. We measured an oxygen consumption of 1.29 ± 0.20 μmol of O_2/min·rat lung, in the absence of CN^-, at 95% oxygen in the polarograph (Fig. 1). Oxygen consumption of lung slices measured in a polarograph was directly related to lung slice masses between 20 and 75 mg. In these and subsequent experiments, 40 mg of lung slices were used.

Oxygen consumption of rat lung slices at 15% O_2 in the absence of CN^- is about 40% of that measured at 95% O_2 (Fig. 1). This occurs because respiration decreases linearly with partial pressure when the P_{O_2} in a heterogeneous tissue such as lung falls below a critical value (38). Even though mitochondrial respiration is maximal at a mitochondrial P_{O_2} of less than 1%, 15% oxygen inhibits respiration in lung tissue slices. This is thought to be due to the inability of oxygen at lowered tensions to diffuse rapidly enough into actively respiring tissues of significant thickness (38). Thinner tissue slices would reduce this problem, but the oxygen consumption measured in 95% oxygen is lower in slices less than 0.5-mm thick, because the proportion of damaged cells in the thinner slices becomes significant (37). Maximal inhibition of respiration in

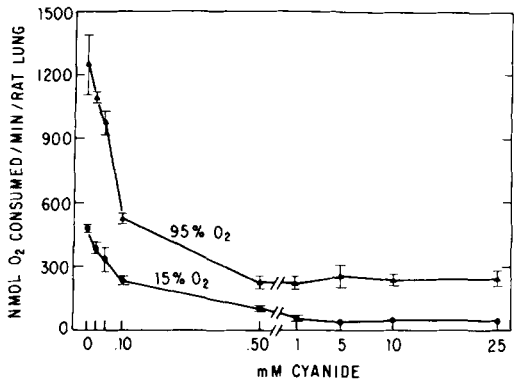

FIG. 1. **Inhibition of lung slice respiration at 15% and 95% oxygen with CN^-.** The oxygen consumption of 40-mg lung slices was measured polarographically as a function of CN^- concentrations. Lung slices were suspended in Krebs-Ringer phosphate buffer at either 15% or 95% saturation with oxygen. n = 5, ± S.E.

lung slices at both 15% oxygen and 95% oxygen was obtained with 1 mM CN^- (Fig. 1). It can also be noted that the CN^- resistant respiration at 95% oxygen is five times greater than at 15% oxygen.

Total and CN^--resistant respiration was measured as a function of P_{O_2} in lung tissue slices of control rats and rats which are resistant to the lethal effect of 100% oxygen following prior exposure to 85% oxygen (15) for 7 days (Figs. 2 and 3). Rats previously exposed to 85% oxygen might adapt by decreasing intracellular oxygen radical production during normoxic and hyperoxic conditions. This could be a factor in resistance to the lethality of 100% oxygen. If this occurs, it should be reflected by rats pre-exposed to 85% oxygen having a lower CN^--resistant respiration compared to controls.

Cyanide-resistant respiration of rat lung slices increases with the P_{O_2} of the incubation medium (Figs. 2 and 3). This was determined using 1 mM CN^-, which maximally inhibits lung slice respiration (Fig. 1). The oxygen consumption of control rat lung slices at 85% oxygen was also measured in the presence of 1 μg/ml of antimycin A or 5 mM azide, giving oxygen consumption rates of 0.37 ± 0.03 and 0.42 ± 0.04 μmol of O_2 consumed/min·rat lung, respectively. Thus, there is no significant difference between CN^--resistant respiration and antimycin A- or azide-resistant respiration of rat lung tissue slices.

The CN^--resistant respiration of rat lung tissue previously exposed to 85% oxygen for 7 days is significantly greater than that of control rats when oxygen consumption is expressed per whole rat lung (Fig. 2) or per mg of rat lung DNA (Fig. 3). This suggests that a decrease in the rate of lung free radical generation during hyperoxia is not a major factor in adaptive or tolerance mechanisms. The increase in both CN^--resistant respiration and total lung tissue oxygen consumption in the 85% O_2-exposed rats is more probably due to a hyperplastic and hypertrophic response of lungs to hyperoxia. This is substantiated by increases of lung mitochondrial-specific cytochrome *c* oxidase and microsomal-specific rotenone-insensitive NADPH-cytochrome *c* reductase activities after rats are exposed to 85% oxygen for 7 days (Table 1). Thus, CN^--resistant respiration in lungs is predominantly a function of P_{O_2} and of alterations in amounts of oxidizable components present in lung tissue.

Hyperoxic conditions will increase the proportion of lung CN^--resistant respiration relative to total tissue oxygen consumption measured in the absence of CN^-. At a dissolved oxygen concentration of 15%, CN^--resistant respiration is 8% of total respiration in both control and 85% oxygen-pre-exposed rats. When dissolved oxygen concentration is 85%, both

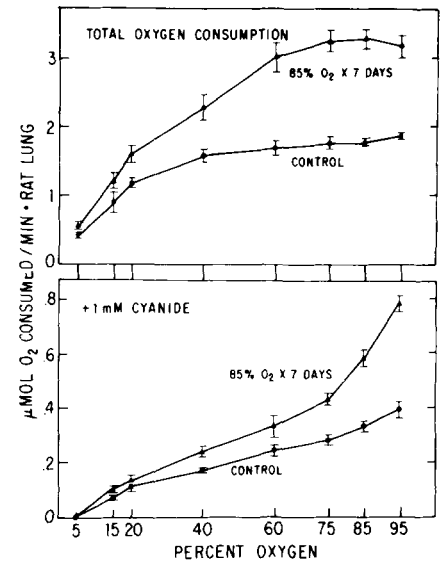

FIG. 2. **Effect of oxygen on rat lung slice total and CN^--resistant respiration.** Lung slices were obtained from control rats and from rats pre-exposed to 85% oxygen for 7 days. Lung slices were suspended in Krebs-Ringer phosphate buffer adjusted to increasing oxygen concentrations, and respiration was measured polarographically. Oxygen consumption is expressed per whole rat lung. n = 5, ± S.E.

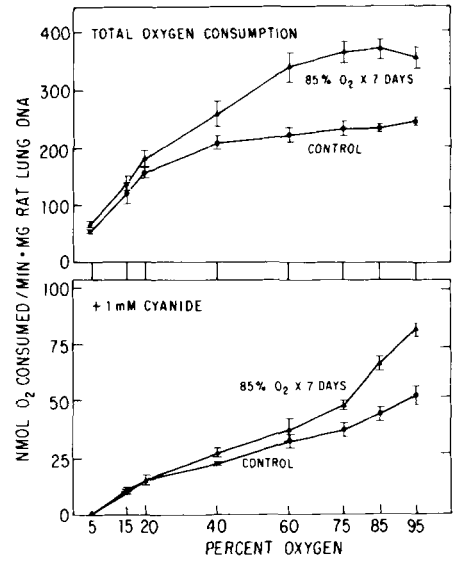

FIG. 3. **Effect of oxygen on rat lung slice total and CN^--resistant respiration.** Lung slices were assayed as described in Fig. 2. Respiration in these lung slices is normalized for oxygen exposure-induced changes in lung cell numbers by expressing oxygen consumption in terms of rat lung DNA.

TABLE I

Lung mitochondrial and microsomal marker enzyme activities following exposure of rats to 85% oxygen for 7 days

Enzyme	Control	85% O_2 × 7 days
Activity in units/lung[a]		
Cytochrome *c* oxidase	1.33 ± 0.06	2.46 ± 0.10
Rotenone-insensitive NADPH-cytochrome *c* reductase	0.147 ± 0.018	0.283 ± 0.012
Activity in units/mg lung DNA[a]		
Cytochrome *c* oxidase	0.18 ± 0.01	0.28 ± 0.02
Rotenone-insensitive NADPH-cytochrome *c* reductase	0.019 ± 0.002	0.032 ± 0.002

[a] 1 unit = 1 μmol/min; n = 5, ± S.E.

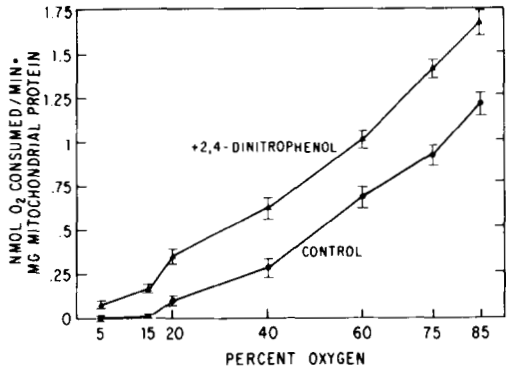

FIG. 4. **Hyperoxia increases CN^--resistant respiration in rat lung mitochondria.** Oxygen consumption in the presence of 1 mM CN^- was measured as a function of oxygen tension in mitochondria isolated from normal rat lungs. Following control measurements, suspensions were made 0.5 mM with 2,4-dinitrophenol and oxygen tensions were readjusted for a second oxygen consumption determination at the P_{O_2} of interest. n = 9, ± S.E.

control and oxygen-pre-exposed tissue-slice CN^--resistant respiration accounts for 18% of total respiration (Figs. 2 and 3).

The Effect of P_{O_2} on CN^--resistant Respiration in Mitochondria—In rat lung mitochondria supplemented with succinate (5 mM) and glutamate (10 mM), addition of 200 to 400 nmol of ADP caused a transition of state 4 respiration[1] to state 3 respiration, allowing calculation of a mean respiratory control ratio of 2.3 ± 0.2 (n = 8, ± S.E.) in the mitochondria used for Fig. 4. The ADP/O ratio for these mitochondrial preparations, which was 2.4 ± 0.4 (n = 8, ± S.E.) and the respiratory control ratio, while comparable to those reported for other rat lung mitochondrial preparations (29, 30) are significantly lower than respiratory parameters reported for liver mitochondria (31). NADH (0.5 mM) did not stimulate state 1 respiration in these mitochondrial preparations, suggesting that organelle permeability was not compromised during isolation.

Oxygen concentrations in excess of 1-2 mm of Hg allow a maximal rate of respiration in mitochondria (39). We examined the effect of P_{O_2} on CN^--resistant respiration of both freshly isolated native and 2,4-dinitrophenol-uncoupled mitochondria, so that the contribution of mitochondrial CN^--resistant respiration to total lung slice CN^--resistant respiration could be determined. Also, Dryer *et al.* (40) have reported that Mn superoxide dismutase is induced in the livers of 2,4-dinitrophenol-fed rats. Mitochondria from these 2,4-dinitrophenol-treated rats generated 60 times more H_2O_2 during state 4 respiration than control rats, leading these authors to conclude that an uncoupled respiratory chain produced an intracellular oxidative stress because of a greater likelihood to monovalently reduce oxygen to O_2^-. Enhanced respiratory chain O_2^- production, following intramitochondrial dismutation of O_2^- to H_2O_2 and O_2, was proposed to be responsible for the elevated rate of H_2O_2 generation measured. We wanted to examine this phenomenon in light of the knowledge that nitrophenyl compounds such as 2,4-dinitrophenol can be metabolized by cellular nitrophenyl reductases to a nitrophenyl free radical (41) which, under aerobic conditions, can then autooxidize to produce O_2^- (42).

Cyanide-resistant respiration in rat lung mitochondria was 0 at 15% oxygen and then increased linearly (r = 0.985) with P_{O_2} until, at 85% oxygen, CN^--resistant respiration was 1.21 ± 0.06 nmol of oxygen consumed/min·mg of protein (Fig. 4). The mitochondrial preparations described in Fig. 4 had a state 3 respiration of 40.8 ± 2.3 nmol of oxygen consumed/min·mg of protein at P_{O_2} of 85%. Thus, CN^--insensitive respiration represented 3% of total mitochondrial oxygen consumption. The same mitochondrial preparations, when treated with 0.5 mM 2,4-dinitrophenol, were maximally uncoupled, since state 3U respiration in the presence of 2,4-dinitrophenol is identical to the mitochondria oxygen consumption measured after ADP addition when 2,4-dinitrophenol is absent. Fig. 4 shows a significant elevation of CN^--resistant respiration, especially at low P_{O_2}, in the 2,4-dinitrophenol-uncoupled mitochondria. This confirms the results of previous investigators (40) that 2,4-dinitrophenol increases free radical production by mitochondrial preparations.

The contamination of mitochondrial preparations by microsomal protein, determined by the method of Sottocasa *et al.* (33), was less than 3.5% of total protein in all preparations, suggesting that measurements of mitochondrial CN^--resistant respiration are not substantially altered by contaminating subcellular organelles.

Characterization of Mitochondrial CN^--resistant Respiration—Azide- and antimycin A-treated mitochondria have rates of oxygen consumption similar to mitochondria inhibited with CN^- (Table II). This cytochrome oxidase-independent respiration is substrate-dependent, since inhibition of NADH-dehydrogenase with rotenone and deletion of succinate, glutamate, or both substrates decreases CN^--resistant respiration. Cyanide-resistant respiration was slightly lowered by addition of exogenous superoxide dismutase, while catalase had a pronounced inhibitory effect (Table II). For this experiment, 4×10^3 units of catalase was added to mitochondrial suspensions containing 1 mM CN^- and, although catalase was partially inhibited by CN^-, 39 ± 6% of the catalase activity remained at the end of the experiment. The decrease in CN^--resistant respiration by catalase is due to diffusion of H_2O_2 out of the mitochondria where catalase yields molecular oxygen and H_2O from H_2O_2, thus decreasing the apparent rate of CN^--resistant oxygen uptake.

The Effect of Nitrophenyl Compounds on Mitochondrial

TABLE II

Inhibition of mitochondrial respiration and mitochondrial CN^- resistant respiration at 85% oxygen

Oxygen consumption was measured polarographically in a 1.5-ml suspension of 2 mg/ml of rat lung mitochondria. The initial concentrations of substrate were succinate (5 mM) and glutamate (10 mM). State 3 respiration was induced by addition of 400 μM of ADP. Under these conditions, the mitochondria had a respiratory control ratio of 2.5 ± 0.2 and a ATP/O ratio of 2.4 ± 0.3 (S.E.).

Condition	Consumption
	nmol O_2 consumed/min·mg mitochondrial protein
State 4 respiration	13.6
State 3 respiration	34.4
+ CN^- (1 mM)	1.53
+ Azide (5 mM)	1.52
+ Antimycin A (50 μg/ml)	1.55
State 3 respiration + CN^- (1 mM)	
+ Rotenone (50 μM)	1.27[a]
− Glutamate	1.19[a]
− Succinate	0.76[a]
− Glutamate, succinate	0.31[a]
+ Superoxide dismutase (60 units)[b]	0.32[a]
+ Catalase (1.5×10^3 units)	0.87[a]
+ Catalase, superoxide dismutase	0.72[a]

[a] $p < 0.025$ for Student's two-tailed t test on paired samples with n = 3.

[b] 10 μl of 2.0 mg·ml^{-1} Mn superoxide dismutase purified from human liver was added.

[1] Definitions of mitochondrial respiratory states are: state 1, respiration in the absence of added ADP and substrate; state 3, respiration in the presence of added ADP and substrate; state 3U, respiration when substrate and uncoupler are added; and state 4, respiration in the absence of ADP when substrate is added.

CN^--resistant Respiration—2,4-Dinitrophenol-uncoupled rat lung mitochondria have a greater CN^--resistant respiration than controls (Fig. 4), especially at low oxygen concentrations. Possible mechanisms explaining this observation could include an increased rate of O_2^- production by the respiratory chain when mitochondria are uncoupled. Also, production of O_2^- by nitro anion-free radicals (generated by the nitroreductase activity present in mitochondria) could account for the increased CN^--resistant respiration reported in Fig. 4. Total and CN^--resistant respiration of mitochondria was measured during incubation with uncouplers which were (2,4-dinitrophenol, (43)) or were not (salicylic acid, (44)) nitrophenyl compounds (Table III). Respiratory characteristics of mitochondria were also examined following treatment with nitrophenyl compounds, which can (nitrofurantoin) or cannot (*o*-nitrobenzoate) be reduced to a nitrophenyl anion free radical by the nitroreductase activity of both rat liver mitochondrial and microsomal fractions (41). Nitro anion-free radicals will reduce oxygen to O_2^- (41), which will be reflected by an increase in CN^--resistant oxygen consumption in treated mitochondria.

Table III shows that 1 mM of 2,4-dinitrophenol and 5 mM salicylic acid uncouple mitochondria and convert "resting" state 4 mitochondria to a state 3-like rate of oxygen consumption (termed state 3U) normally seen when an excess of ADP is present. Nitrofurantoin and *o*-nitrobenzoate do not significantly change the rate of state 4 oxygen consumption, thus they do not uncouple mitochondria. CN^--resistant respiration of state 3 mitochondria is 1.43 nmol of O_2 consumed/min·mg of protein (Table III). 2,4-Dinitrophenol and nitrofurantoin, which can be metabolized to the nitro anion-free radical, increase CN^--resistant respiration in a dose-dependent fashion. *o*-nitrobenzoate, a nitrophenyl compound not reducible to the nitro anion-free radical, has no effect on mitochondrial CN^--resistant respiration. Salicylic acid, which uncouples mitochondria, contains no reducible nitro groups capable of transferring electrons directly to oxygen and does not increase CN^--resistant respiration. Thus, in contrast to other investigators (40), we find no correlation between uncoupling of respiration in mitochondria and O_2^- formation by these organelles.

TABLE III

The effect of nitroaromatic compounds on rat lung mitochondrial respiration and mitochondrial CN^--resistant respiration at 85% oxygen

Oxygen consumption was measured as described in Table II. Under these conditions, the mitochondria had a respiratory control ratio of 2.5 ± 0.3 and a ATP/O ratio of 2.4 ± 0.3.

Condition	nmol O_2 consumed/min·mg mitochondrial protein
State 4 respiration	13.2
State 3 respiration	33.8
State 4 respiration	
+ 2,4-dinitrophenol (0.5 mM)	34.5[a]
+ *o*-nitrobenzoate (1 mM)	12.4[b]
+ nitrofurantoin (5 mM)	12.6[b]
+ salicylic acid (5 mM)	33.4[a]
State 3 respiration + CN^- (1 mM)	1.43[a]
+ 0.5 mM 2,4-dinitrophenol	2.27[a]
+ 2.0 mM 2,4-dinitrophenol	2.61[a]
+ 5.0 mM 2,4-dinitrophenol	3.34[a]
+ 0.5 mM nitrofurantoin	1.67[a]
+ 2.0 mM nitrofurantoin	2.60[a]
+ 5.0 mM nitrofurantoin	3.07[a]
+ 0.5 mM *o*-nitrobenzoate	1.37[b]
+ 5.0 mM *o*-nitrobenzoate	1.34[b]
+ 0.5 mM salicylic acid	1.43[b]
+ 2.0 mM salicylic acid	1.43[b]
+ 5.0 mM salicylic acid	1.46[b]

[a] $p < 0.025$ for Student's two-tailed t test on paired samples with $n = 3$.

[b] Not significant for control.

DISCUSSION

Superoxide dismutases, ubiquitous among oxygen-utilizing cells, protect an organism from O_2^- and the highly reactive secondary species of oxygen which can arise from further reactions to O_2^-. While an increased production of oxygen radicals in lung cells has been postulated to be a major factor in the etiology of lung damage during hyperoxia (1), this has never been proven. In this report, we provide evidence for an association between hyperoxia and the increased production of partially reduced species of oxygen by lung tissue and lung mitochondria. The induction of Mn superoxide dismutase in rat lungs following hyperoxic exposure (5) is consistent with the observation herein that hyperoxia increases mitochondrial oxygen radical production, since in rats Mn superoxide dismutase is a mitochondria-specific enzyme (45, 46). The increase in rat lung CN^--resistant respiration during hyperoxia also derives from cellular sources, in addition to mitochondria. Thus, it is not surprising that CuZn superoxide dismutase, which resides in both the cytoplasm and mitochondria (46) and accounts for the majority of rat lung superoxide dismutase activity (5), is also induced by hyperoxia as an adaptive response (47).

The greater CN^--resistant respiration of 85% oxygen-preexposed rats relative to controls suggests that rats previously exposed to 85% oxygen for 7 days are resistant to 100% oxygen toxicity because of induced antioxidant enzymes, rather than an adaptive decrease in intracellular O_2^- production. The increase in total tissue oxygen consumption and the CN^--resistant respiration of rat lungs previously exposed to 85% oxygen is likely due to both the hyperplasic and hypertrophic response of lung cells to hyperoxia. Electron microscope morphometric measurements of lungs have shown both cell number and mean cell volumes in the alveolar region (measured in rats of the same species and age described herein) to increase following inhalation of 85% oxygen for 7 days (5). The hyperplasic response of rat lungs to hyperoxia is also confirmed by the knowledge that the DNA content of the rat lungs reported in Figs. 1–3 was 7.51 ± 0.10 mg/lung, and following exposure to 85% O_2 for 7 days was 8.80 ± 0.10 mg of DNA/lung ($n = 5$, ± S.E., $p < 0.025$). Lung mitochondrial-specific cytochrome *c* oxidase and microsomal rotenone-insensitive NADPH-cytochrome *c* reductase activities also increase following oxygen exposure of rats (Table I). This supports the microscopic observation of hyperoxia-induced lung cell hypertrophy (5). These enzyme activities increased not only on a whole lung basis but also when normalized by lung DNA content, suggesting that on the average, the 85% oxygen-exposed lung cells contained greater amounts of mitochondrial and endoplasmic reticulum protein. Thus, rates of H_2O_2 and O_2^- generation in lungs indicated by measurement of CN^--resistant respiration are primarily a function of P_{O_2} and of lung cell proliferation and hypertrophy.

From the data reported herein, the contribution of mitochondria to rat lung CN^--resistant respiration can be estimated. The cytochrome *c* oxidase activity of the rat lungs used for mitochondrial isolations and CN^--insensitive respiration measurements (Fig. 4) was 0.98 ± 0.18 units/lung. The lung mitochondrial preparations had 0.029 ± 0.002 units of cytochrome *c* oxidase/mg of mitochondrial protein, with 8.11 ± 0.37 mg of mitochondrial protein isolated/lung. Thus, there was an average 24% recovery of mitochondrial cytochrome *c* oxidase activity from the rat lungs. The average rat lung can also be estimated to contain 34 mg of mitochondrial protein.

Under hyperoxic conditions (such as 85% oxygen), the contribution of mitochondria to pulmonary CN^--resistant respiration is 51 nmol of O_2 consumed/min, while the CN^--resistant respiration of a whole rat lung is 330 ± 20 nmol of O_2/min. In hyperoxia, the mitochondrial contribution to whole lung CN^--insensitive respiration is 15%. Figs. 2 and 4 show that mitochondria contribute little to the CN^--resistant respiration measured under normoxic conditions.

Boveris *et al.* (48) demonstrated that rat liver mitochondria, along with microsomes, peroxisomes, and soluble enzymes, are intracellular sources of H_2O_2. Dionisi *et al.* subsequently reported that O_2^- was the direct precursor of all mitochondrial H_2O_2 (49), according to Equation 3. There are multiple possible loci of mitochondrial O_2^- generation. Various investigators have proposed that autooxidation of ubisemiquinone (50), the flavin semiquinone of NADH dehydrogenase (24, 51, 52), and cytochrome b_{566} (27), are the sources of mitochondrial O_2^-. These sites of O_2^- generation were identified using rat liver and beef heart submitochondrial particles washed free of contaminating superoxide dismutase. The quantitative contribution of these various components to O_2^- generation may vary depending upon the organ and species from which the mitochondria were isolated. Under normoxic *in vitro* conditions, rat liver mitochondria fully supplemented with substrates account for 14% of the total organ H_2O_2 production (48). This is similar to our estimation of 15% as the mitochondrial contribution to rat lung CN^--resistant respiration under hyperoxic *in vitro* conditions. Most nonenzymatic sources of cellular O_2^- production (*i.e.* autooxidation of mitochondrial respiratory chain components) should react close to first order with respect to oxygen concentration. Boveris and Chance (50) have demonstrated that hyperbaric oxygen increases rat liver H_2O_2 production linearly as a function of oxygen pressure. Thus, as intracellular oxygen concentration increases, so will the rate of O_2^- production, but not necessarily the proportion of O_2^- produced by particular cytosolic sources. Equation 4, where $R\cdot$ is any autooxidizable molecule, will describe the oxygen dependence of mitochondrial O_2^- production.

$$dO_2^-/dt = k\,[O_2]\,[R\cdot] \tag{4}$$

We have demonstrated that CN^--resistant respiration is a good indicator of the effect of pO_2 on the production of partially reduced species of oxygen in lung tissue. Hassan and Fridovich (13, 14) have reported that CN^--resistant respiration measurements in bacteria also are related to the intracellular production of O_2^- and H_2O_2. Cyanide-resistant respiration, however, does not serve as an absolute measure of cellular O_2^- and H_2O_2 production in lung tissue. First, the rate of O_2^- production by isolated mitochondria depends on the metabolic state. For example, the greater the reduction of respiratory chain components [such as during state 4 respiration (43)], the greater the rate of O_2^- production (22). Thus, *in vitro* measurements of mitochondrial oxygen radical production may not accurately reflect *in vivo* generation rates. Second, blockage of respiratory chain electron flow by antimycin A or inhibition of cytochrome *c* oxidase with CN^- increases respiratory chain component reduction (43), resulting in mitochondrial O_2^- generation rates greater than what may occur *in situ* under various O_2 concentrations (22). The quantitation of lung O_2^- production could conversely be underestimated when using CN^- as an electron transport inhibitor, since CN^- partially inhibits the production of O_2^- by bovine heart mitochondria (51). Finally, intracellular dismutation of O_2^- to H_2O_2 and O_2 by superoxide dismutase and metabolism of H_2O_2 to H_2O and O_2 by peroxidases could result in as much as a 4-fold underestimation of lung O_2^- generation and a 2-fold underestimation of H_2O_2 generation by polarographic measurements of CN^--resistant respiration, where $R\cdot$ is any autooxidizable molecule (Equations 5–7).

$$4O_2 + 4R\cdot \longrightarrow 4O_2^- \tag{5}$$

$$4O_2^- \xrightarrow{\text{superoxide dismutase}} 2H_2O_2 + 2O_2 \tag{6}$$

$$2H_2O_2 \xrightarrow{\text{catalase}} O_2 + 2H_2O \tag{7}$$

Only oxygen reduced to H_2O or oxygen species covalently reacting with cellular constituents will be recorded polarographically as CN^--resistant respiration if the reactions in Equations 5–7 occur. Thus, CN^--resistant respiration serves as an approximate quantitation of the partial reduction of oxygen by cells and reflects not only the production of O_2^- and H_2O_2 but also the oxidation of substrates such as lipids, amino acids, and nucleotides.

Catalase reduced mitochondrial CN^--resistant respiration from 1.53 nmol of O_2/min·mg of mitochondrial protein to 0.87 nmol of O_2/min·mg of protein (Table II). This catalase-induced CN^--resistant respiration decrement of 0.66 nmol of O_2/min·mg of mitochondrial protein implies that 1.32 nmol of H_2O_2/min·mg of mitochondrial protein was diffusing extramitochondrially, using Equation 7. If one assumes that all mitochondrial H_2O_2 derives from O_2^- dismutation (22, 25, 49), it can be calculated from Equation 6 that mitochondria incubated at 85% oxygen generate about 2.6 nmol of O_2^-/min·mg of mitochondrial protein. This value agrees well with measurements of O_2^- production by beef heart submitochondrial particles (52) and is more than twice the CN^--resistant respiration measured in isolated lung mitochondria; 1.25 nmol of O_2 consumed/min·mg of mitochondrial protein. These results also show that H_2O_2 diffuses freely out of mitochondria during hyperoxia. The physiological significance of this observation is uncertain, however, because 1 mM CN^- can inhibit mitochondrial catalase. Intramitochondrial catalase, GSH, and glutathione peroxidase activity was not examined under conditions of these experiments.

Table III shows that uncoupling does not increase mitochondrial CN^--resistant respiration, and reconfirms that nitrophenyl anion radical products of mitochondrial nitroreductase can directly reduce oxygen. Uncoupling with nonreducible compounds such as salicylate (53) will cause development of a state 4-like reduction of respiratory components and could increase the rate of O_2^- production by this more reduced respiratory chain. Uncoupling by salicylate either does not enhance mitochondrial O_2^- production or results in O_2^- production below the sensitivity of CN^--resistant respiration measurements (Table III). These observations do not rule out the possibility that some lesion is produced when mitochondria are uncoupled, which may destabilize the respiratory chain and enhance autooxidation of respiratory chain components, ultimately increasing the specific activity of mitochondrial O_2^- production. If this phenomenon occurs, the resultant O_2^- generation is below the sensitivity of CN^--resistant respiration measurements.

Our data show that hyperoxia will increase the steady state concentrations of O_2^- and H_2O_2 in lungs, which in turn can react according to Equations 2 and 3 and yield $OH\cdot$. Many lines of evidence indicate that reduced oxygen species are capable of initiating lipid peroxidation, enzyme inhibition, DNA strand breakage (12), and in the case of lungs, ultimately lead to the development of pulmonary edema due to capillary endothelial cell damage (54, 55).

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