Hyperoxia: Influence on Lung Mechanics and Protein Synthesis

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ABSTRACT We studied the time-course of the influence of in vivo hyperoxia on lung mechanics and on protein synthesis. After 24 h of exposure to greater than 98% O₂ at 1 atm there were no alterations in descending pressure-volume curves (air or saline) of lungs excised from O₂-exposed rats compared to control rats. After 48 h of hyperoxia there was a decrease in lung compliance.

To study protein synthesis, as indicated by L-[U-¹⁴C] leucine incorporation into protein, lung slices were incubated with L-[U-¹⁴C]leucine and surface-active material then obtained by ultracentrifugation of lung homogenates. We measured radioactivity in total protein and in protein in the surface-active fraction. There were no alterations in incorporation after 12 h of hyperoxia. After 24 h of hyperoxia there were significant decreases ($P < 0.05$) in L-[U-¹⁴C]leucine incorporation into total protein and into protein of the surface-active fraction. After 48 h of hyperoxia incorporation into protein of the surface-active fraction was decreased to a greater extent than incorporation into total protein, $63 \pm 4\%$ and $75 \pm 5\%$, respectively, ($P < 0.025$).

These studies show that hyperoxia produces a major decrease in protein synthesis, including synthesis of protein in a surface-active fraction, before the onset of any detectable changes in the static compliance of excised lungs.

INTRODUCTION

It is widely recognized that oxygen in high concentration is toxic to the lung. Prolonged exposure of animals to oxygen at partial pressures greater than are present in room air alters the lung's mechanical properties (1). These changes suggest that the surface forces of terminal lung units have increased (2). Several hypotheses have been advanced to explain these mechanical changes including the suggestion that they could be due to an

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oxygen-induced decrease in the synthesis of lung surface-active lipoprotein (1). Unfortunately, little is actually known about the influence of hyperoxia on lung biosynthetic processes. Even less is known about the time relationship between alterations in the synthesis of substances likely to be components of surface-active material and alterations in lung mechanics. These considerations led us to ask two questions. First, is in vitro protein synthesis, as measured by incorporation of L-[U-¹⁴C] leucine into protein, altered in lungs from rats exposed to hyperoxia? If so, does this alteration in protein synthesis, with particular reference to those proteins found in a surface-active lung fraction, precede or follow detectable changes in the lung's mechanical properties?

MATERIALS

Animals. We used male Dublin Sprague-Dawley derived rats (Flow Research Animals, Inc., Dublin, Va.) which were allowed food (Wayne Lab-Blox, Allied Mills, Inc., Chicago, Ill.) and water ad lib. For exposure to oxygen they were maintained in cages and supplied by humidified oxygen from a wall outlet at 10 liters/min. The O₂ and CO₂ concentrations were measured every 6 h during an initial series of experiments and then once during every experiment using a gas chromatograph (Fisher-Hamilton Gas Partitioner, Fisher Scientific Products, Silver Spring, Md.). The O₂ concentration was never found to be lower than 98% nor the CO₂ concentration greater than 0.4%. Control rats were kept in identical adjacent cages which were supplied by humidified compressed air from a wall outlet at 10 liters/min. The animals were sacrificed by exsanguination after the intraperitoneal injection of sodium pentobarbital, 30 mg/kg.

Incubation of lung slices. Lungs were removed as previously described (3) and sliced at 0.3 mm thickness using a McIlwain tissue chopper (Brinkman Instruments, Westbury, N. Y.). Incubations were performed at 40°C in rubber-capped flasks containing Waymouth medium (Grand Island Biological Co., Grand Island, N. Y.), and L-[U-¹⁴C] leucine (New England Nuclear Corp., Boston, Mass.). The gas phase was 95% O₂-5% CO₂ and the flasks were shaken at 90 oscillations/min. In some studies we performed "pulse-chase" experiments to determine the influence of hyperoxia on the degradation of the newly synthesized protein. To do this we incubated about 100 mg of lung slices with L-[U-¹⁴C]leucine at 40°C for 10 min but used Earle's Balanced Salt Solution (2.5 ml) (Grand Island Biological Co.) as the medium. The flasks (3 from each O₂ and control rat) were then chilled on ice, the medium removed and

the slices washed quickly with Waymouth medium. These slices were then reincubated in Waymouth medium (which contains 0.38 μmol of L-[^{14}C]leucine without radioactive leucine at 40°C for 0 or 60 min.

Isolation of a surface-active lung fraction. In experiments where we isolated surface-active material, the reactions were stopped by chilling and all subsequent procedures performed at 4°C. The medium was removed, the slices washed quickly with cold 0.15 M NaCl and homogenized by 30 passes of a motor driven Teflon pestle in a glass homogenizing vessel. A sample of the homogenate was saved to determine the incorporation of radioactivity into crude lung protein and the remainder was centrifuged at 300 *g* for 10 min. The remainder of the isolation procedure was as previously described (4) and represents a modification of the procedure described by Klein and Margolis (5).

Assay for radioactivity. The tissue proteins and the proteins in the surface-active lung fraction were precipitated with trichloroacetic acid (TCA),¹ extracted with lipid solvents and hot TCA, and assayed for radioactivity and protein content as previously described (6, 7). The hot TCA-soluble material from the crude extracts was saved for measurement of DNA content relative to the amount of acid-insoluble radioactivity.

Nature of acid-soluble radioactivity. The acid-soluble material from several incubation mixtures was collected, separated from the TCA, subjected to paper chromatography and assayed for radioactivity as previously described (8). We used material from incubations of tissue from air and O₂-exposed rats separately.

Chemical determinations. Protein was measured on the material not soluble in cold or hot TCA or in lipid solvents but which was soluble in 0.2 M NaOH. Crystallized bovine serum albumin (Mann Research Labs., Inc., N. Y.) served as standard (9). To obtain values for protein to DNA and RNA to DNA ratios, these substances were separated and isolated in whole tissue homogenates by the alternate procedure of Schneider (10). DNA was measured using calf thymus DNA as the standard (Mann Research Labs. Inc.). RNA was measured using yeast RNA as the standard (General Biochemicals, Div. Mogul Corp., Chagrin Falls, Ohio).

To determine the amount of free leucine in the lung tissue 500 mg (wet weight) samples of lung were homogenized in 2.5 ml of cold water. Cold TCA was added to a final concentration of 10%, the mixture was allowed to stand on ice for 30 min and then the mixture was centrifuged. The supernatant material was extracted with ethyl ether to remove the TCA. This extraction was continued until the pH of the aqueous phase rose to 6.0. Leucine was measured in the acid-soluble material by ion exchange chromatography using a Beckman Model 120C amino acid analyzer (Beckman Instruments, Inc., Fullerton, Calif. (11). DNA and RNA were determined on the cold TCA-insoluble sediment by the alternate method of Schneider (10). Lung wet to dry weight ratios were measured by weighing lightly blotted lung slices (wet weight) and then reweighing these slices every 24 h after placing them at 60°C. The weight which did not change after two consecutive measurements 24 h apart was taken as the dry weight.

Pressure-volume measurements. The pressure-volume (P-V) measurements were made as described by Johnson,

Permutt, Sipple, and Salem (12). The lung was completely degassed in a vacuum jar. The lung was then inflated by means of a calibrated syringe. Transpulmonary pressure was measured by a Statham PM5 pressure transducer (Statham Laboratories, Hato Rey, Puerto Rico). The air volume at a transpulmonary pressure of 30 cm H₂O was considered as maximum lung volume. The lung appeared completely inflated at this point. Deflation was carried out, after two inflations to 30 cm H₂O, to predetermined transpulmonary pressures, i.e. 20, 15, 10, 5, and 0 cm H₂O. The lung volume at each pressure was recorded. In some experiments P-V measurements were made using saline to inflate the lung (13).

Statistical calculations. Table II was derived from Table I. To do this we set the mean protein-specific radioactivity of the air exposed rats at 100% for each time of exposure. Each protein-specific activity from the air-exposed animals was then expressed as a percentage of this mean value and the SEM calculated from these values. This same mean protein-specific activity also served as 100% for the oxygen-exposed rats for each time of exposure. Each protein-specific activity from the oxygen-exposed animals was then expressed as a percentage of this figure and the SEM calculated from these values. The weight changes in individual animals with air or oxygen exposure were analyzed using a paired *t* test (14). The significance of the differences between other values from these animals were tested by an unpaired *t* test analysis (14). SE were calculated by conventional techniques. The *P* values have the usual connotation as an index of probability.

RESULTS

Influence of in vivo oxygen exposure for 48 h on L-[U- ^{14}C]leucine incorporation into total protein and on degradation of newly formed radioactive protein. The protein-specific radioactivity of total acid-insoluble material is greater in lungs from air-exposed animals than in lungs from oxygen-exposed animals (Fig. 1a). This difference assumes statistical significance after a 1 h incubation ($P < 0.01$). When the acid-insoluble radioactivity is expressed per mg of DNA the differences are also significant after 1 h incubation (Fig. 1b) ($P < 0.01$). Measurements on the free leucine content of lung tissue revealed less free leucine in the O₂-exposed rats (Table V). When incorporation rates are corrected for the size of the pool of free leucine the differences in acid-insoluble radioactivity between lungs from air and O₂-exposed rats became greater. The acid-insoluble radioactivity of the O₂-exposed rats was 65% of the air-exposed rats at 30 min ($P < 0.025$), and 51% of the air-exposed rats at 60 min ($P < 0.005$). In other experiments where we "pulse-labeled" lung slices with L-[U- ^{14}C]leucine the lungs from air- and O₂-exposed rats had a virtually identical fall in protein-specific radioactivity during the post-pulse hour ($14.3 \pm 3.1\%$ and $15.4 \pm 4.0\%$, respectively).

Time-course of the influence of in vivo oxygen exposure on the P-V relationships of excised lungs. Fig. 2 shows the influence of exposure to hyperoxia on the deflation P-V curve. After 24 h of exposure there were

¹ Abbreviations used in this paper: P-V, pressure-volume measurements; RER, rough endoplasmic reticulum; TCA, trichloroacetic acid.

FIGURE 1 Influence of in vivo oxygen exposure on L-[U-¹⁴C]leucine incorporation into protein. In each experiment three replicate flasks from O₂ and control rats containing about 200 mg of slices lung and 10 μ l of L-[U-¹⁴C]leucine (9.8×10^{-4} μ mol; sp act 255 μ Ci/ μ mol) were incubated in Waymouth medium. Mean $\pm$ SEM are given.

no differences in the P-V curves between the two groups when air was used to inflate the lungs (Fig. 2a) or when saline was used to inflate the lungs (not shown, five rats in each group). After 48 h of exposure the P-V curve of lungs from oxygen-exposed rats was shifted to the right when compared to that of lungs from air-exposed rats (Fig. 2b).

Time-course of the influence of in vivo hyperoxia on [U-¹⁴C]leucine incorporation into total protein and into protein in a surface-active lung fraction. We found no differences in protein-specific radioactivity after 12 h of in vivo hyperoxia (Table I). After 24 h of exposure the protein-specific radioactivity was lower in both the protein of the whole tissue homogenate and in the protein of the surface-active fraction in lungs from rats exposed to hyperoxia (Table I). Thus, we found substantial changes in [¹⁴C]leucine incorporation into protein before we detected any changes in the lung P-V characteristics. After 48 h of hyperoxia there was a greater decrease in the protein-specific radioactivity of the surface-active fraction than of the protein of the whole tissue homogenate (Table I). This latter point is shown in Table II where these results are ex-

pressed as a percentage of the mean protein-specific radioactivity using the mean figures for the air-exposed rats as 100% for each time interval.

Influence of hyperoxia on animal weight. There were no differences in initial weight between groups of oxygen- or air-exposed animals (Table III). Both groups gained equal amounts of weight up to 24 h of exposure. Thereafter, the rats exposed to hyperoxia lost weight. The rate of weight gain decreased in air-exposed rats.

Influence of in vivo L-tryptophan on L-[U-¹⁴C]leucine incorporation into protein. We have previously shown that tryptophan reverses the decrease in protein synthesis by lungs from rats starved for 48–72 h (4). In this present study, although the animals were allowed food and water ad lib., those exposed to oxygen for 48 h lost weight (Table III). We therefore sought to determine if the decrease in protein synthesis in animals exposed to oxygen for 48 h would be altered by the administration of tryptophan. Unlike its effect on food deprived but air-exposed animals (4), tryptophan did not reverse the decrease in protein synthesis by lungs from oxygen-exposed rats (Table IV).

FIGURE 2 Influence of in vivo oxygen exposure on air deflation P-V curves of excised lungs. Each curve represents five animals. Mean $\pm$ SEM are given.

TABLE I
Influence of In Vivo Oxygen Exposure on Amino Acid Incorporation into Total Protein and into Protein of a Surface-Active Fraction by Rat Lung Slices

Exposure	Atmosphere	Protein-specific radioactivity		
		Total	Surface-active fraction	
<i>h</i>		<i>cpm/mg protein</i>		
12	Air	1199 $\pm$ 84 (4)	1952 $\pm$ 98 (4)	<i>P</i>
	O ₂	1103 $\pm$ 72 (4)	1931 $\pm$ 155 (4)	
		NS	NS	
24	Air	1101 $\pm$ 62 (8)	1992 $\pm$ 178 (6)	<i>P</i>
	O ₂	812 $\pm$ 46 (6)	1559 $\pm$ 125 (4)	
		0.005	0.05	
48	Air	1102 $\pm$ 84 (4)	1847 $\pm$ 82 (4)	<i>P</i>
	O ₂	832 $\pm$ 34 (7)	1164 $\pm$ 87 (7)	
		0.005	0.001	

In each experiment a single flask containing about 500 mg of sliced lung tissue in 5.0 ml of medium was incubated for 60 min with 25 μ liter of L [U-¹⁴C] leucine (2.4×10^{-3} μ mol; sp act 306 μ Ci/mol). Values in parentheses indicate number of animals. Mean $\pm$ SEM are given. NS, *P* > 0.05.

Nature of acid-soluble and acid-insoluble radioactivity. All the acid-soluble radioactivity from both oxygen-exposed and control rats has the same *R_f* as known leucine in two different solvent systems (8).

Influence of in vivo hyperoxia on biochemical parameters in lung. Of those parameters measured (Table V) only the free leucine content of lung tissue was significantly different between the two groups.

DISCUSSION

The present study has shown that lung slices from rats exposed to hyperoxia and incubated with [¹⁴C]leucine have a lower protein-specific radioactivity in the protein of the whole tissue homogenate and in the protein of the surface-active fraction than lung slices from rats exposed to compressed air. These differences become larger when corrected for free leucine content of lung tissue indicating they are not due to differences in the total level of free leucine in these tissues. However, this does not exclude the possibility that leucine pools may be compartmentalized and hence that the protein precursor pool might be different in control and O₂-exposed rats (15). Indeed, a recent study has indicated that in liver, free intracellular valine exists as two distinct pools (16). One pool appears to be independent of external valine while the other pool equilibrates rapidly with the suspending medium and appears to be in continuity with sites of protein synthesis. Such in-

TABLE II
Relative Effect of In Vivo Oxygen Exposure on Amino Acid Incorporation into Total Protein and into Protein of a Surface-Active Fraction

Exposure	Atmosphere	Percent of control		
		Total radioactive protein	Surface-active fraction radioactive protein	
<i>h</i>	Air	100±7	100±2	
12	O ₂	92±5	99±5	NS
	Air	100±6	100±9	
24	O ₂	74±5	78±6	NS
	Air	100±12	100±1	
48	O ₂	75±5	63±4	0.024

The values in this table are derived from the protein-specific radioactivities in Table I. See text for an explanation. *P* is given for the difference between the effect of oxygen on incorporation into total protein and into protein in the surface-active fraction.

formation is not available for free amino acid pools in the lung. However, the kinetics of [¹⁴C]leucine transport from the suspending medium to acid-soluble material in lung slices and then into protein indicates that there is a very rapid equilibration of leucine in the medium with leucine serving as a precursor for protein synthesis (7). Therefore, the use of an in vitro system of lung tissue would, as in other tissues (17), diminish any potential inequality in intracellular amino acid pools. The latter would rapidly equilibrate with the much higher concentrations of amino acid in the incubating medium. The demonstration that the acid-soluble radioactivity is leucine in both the oxygen-exposed and control animals indicates this radioactive substrate did not undergo conversion to other metabolites before being incorporated into protein and hence was not diverted into other pathways to any serious degree. The similar deg-

TABLE III
Influence of Oxygen on Animal Weight

Exposure	Air		O ₂	
	Initial	Final	Initial	Final
<i>h</i>	<i>g</i>		<i>g</i>	
12	253±13 (4)	265±10 (4)	252±4 (4)	264±6 (4)
24	262±15 (8)	275±15 (8)	263±15 (6)	272±14 (6)
48	271±15 (4)	283±60 (4)	282±23 (7)	268±21 (7)

Values are mean±SEM. Values in parentheses indicate number of animals.

TABLE IV
Influence of In Vivo Tryptophan on Amino Acid Incorporation into Total Protein and into Protein of a Surface-Active Fraction by Lung Slices

Tryptophan	Protein-specific radioactivity			
	Total		Surface-active fraction	
	+	-	+	-
	<i>cpm/mg protein</i>			
Air	805±58 (4)	829±58 (5)	1316±229 (4)	1327±11 (5)
O ₂	602±36 (5)	688±27 (5)	786±27 (5)	900±86 (5)
<i>P</i>	0.025	0.025	0.05	0.95

Animals were exposed to O₂ or air for 48 h. In each experiment a single flask containing about 500 mg of sliced lung tissue in 5.0 ml of medium was incubated for 60 min with 25 μ liter of L-[U-¹⁴C] leucine (2.4×10^{-3} μ mol; sp act 262 μ Ci/mol). Tryptophan treated rats received that amino acid (29 mg/100 g of body weight) in 0.75 ml of 0.2 M NaOH intraperitoneally 45 min before sacrifice. Control animals were given an equal amount of NaOH without tryptophan. Values in parentheses indicate number of animals. Mean±SEM are given.

radation rates indicate the differences in protein-specific radioactivity are not due to differences in degradation over the time studied.

Food deprivation decreases protein synthesis by some tissues (18) including the lung (4). This effect in lung and in other tissues is reversed by the in vivo administration of tryptophan. Since in the present study the

TABLE V
Influence of In Vivo Oxygen Exposure on Chemical Composition on the Lung

Parameter	Air	O ₂ , 48 h	<i>P</i>
% of dry weight	19.2±0.4 (10)	18.0±0.6 (7)	NS
mg DNA/100 mg tissue	0.15±0.06 (11)	0.14±0.04	NS
mg protein/100 mg tissue	4.99±1.05 (11)	4.42±0.75 (11)	NS
mg RNA/100 mg tissue	0.53±0.10 (11)	0.48±0.12 (11)	NS
Protein/DNA	36.0±3.7 (11)	32.1±3.0 (11)	NS
RNA/DNA	3.69±0.6 (11)	3.45±0.9 (11)	NS
μ mol leucine/mg DNA	49.6±4.3 (5)	37.3±4.4 (5)	0.05

Values in parentheses indicate number of animals. Mean ±SEM are given. NS, *P* > 0.05.

oxygen-exposed rats ate less and lost weight, the differences in [^{14}C]leucine incorporation into protein could have been due to food deprivation rather than to a more direct effect of oxygen on protein biosynthesis. We think this is unlikely for two reasons. First, we noted changes in protein synthesis after 24 h of oxygen exposure, before weight loss occurred. Secondly there was a complete failure of tryptophan to reverse the decrease in leucine incorporation in the present study after weight loss had occurred (48 h of oxygen exposure).

Our finding that [^{14}C]leucine incorporation into protein present in a surface-active fraction is depressed to a greater extent than its incorporation into total protein is intriguing. One explanation is simply that oxygen exerts a differential degree of inhibition on [^{14}C]leucine incorporation into lung proteins, and that mere chance was operative in the greater depression noted in the synthesis of protein in the surface-active fraction. A second possibility, which might act in concert with the first, is that oxygen increases the rate of degradation of protein in the surface-active fraction relative to protein in the total lung homogenate thereby lowering its specific radioactivity. A third possibility is that oxygen exerts a more profound depressing effect on the synthesis of lung secretory protein, i.e. pulmonary surfactant, than it does on the synthesis of structural and nonsecretory protein. The present study does not allow us to choose among these possibilities. However, it is of some interest to consider the last explanation in the light of available information. First, it is generally accepted that secretory and nonsecretory proteins are synthesized on different intracellular organelles, the rough endoplasmic reticulum (RER) and free ribosomes, respectively, (19). Secondly, the integrity of the RER is dependent on the lipid-rich membranous endoplasmic reticulum whereas the free ribosomes are connected together by strands of messenger ribonucleic acid (mRNA). Based on the known disruptive effect of oxygen on lipid membranes (20), it is conceivable that the lipid-rich endoplasmic reticulum could be more susceptible to hyperoxia than the polysomes. Finally, it has been reported that oxygen exposure denudes the RER in the granular pneumocyte of ribosomes (21).

It is difficult to compare our results on protein synthesis with the influence of hyperoxia on other biosynthetic properties of the lung since the other studies used hyperbaric oxygenation. Thus, Newman and Naimark (22) reported that hyperoxia enhanced the *in vivo* incorporation of radioactive palmitate into lung phospholipids. McSherry and Gilder (23) found that the incorporation of radioactive palmitate into phospholipid per gram of lung decreased with increasing exposure to hyperbaric oxygen. Unfortunately, they did not indicate the degree of edema in these lungs and hence, one can-

not make a meaningful interpretation of the incorporation figures expressed per gram of wet lung.

Beckman and Weiss (2) have shown that the lung compliance of rats exposed to 100% oxygen at 1 atm for 60–66 h is lower than that of animals exposed to room air. They concluded that this change in compliance was predominantly due to an increase in surface forces. It seems clear from the present work that in rats exposed to hyperoxia there is a decrease in protein synthesis before there is a detectable fall in compliance when measured using either air or saline to inflate the lungs. Our failure to detect mechanical changes after 24 h of hyperoxia is consistent with a careful stereological study of the air-blood barrier in rats, which failed to reveal any differences in its thickness after 24 h of hyperoxia (24).

The next obvious question is how do we relate the decrease in protein synthesis to the mechanical changes in lungs from rats exposed to hyperoxia? The answer to this question depends, at least in part, on how one interprets the finding of decreased synthesis of proteins in the surface-active fraction of lung. This fraction probably contains some proteins which are not components of the surface-active lipoprotein and hence one could rightly argue that we cannot really be sure that [^{14}C]leucine incorporation into this specific protein is decreased. We cannot disprove this contention. However, the decreased incorporation into the surface-active fraction is associated with a decrease in the size but no change in number of the lamellar bodies (25). Substantial evidence indicates that lamellar bodies are storage sites for surfactant. We view this decrease in size as complementary evidence to the incorporation data indicating decreased synthesis of the apoprotein of the surface-active lipoprotein. If this interpretation of our data is accepted then the relationship between hyperoxia, altered protein synthesis, and changes in lung compliance can be explained at least in part. Decreased synthesis of lung surfactant would result in a decreased amount of this material available for secretion onto the alveolar surface, resulting in increase in surface forces, and hence a decrease in compliance. The time-course of these biosynthetic and mechanical changes is consistent with the postulated half-life of the phospholipid component of lung surfactant (26). An increase in alveolar surface forces could also produce pulmonary edema (27), which is an additional constant finding in oxygen toxicity (1). Thus, a decrease in surfactant synthesis could account for the mechanical and histological changes seen in oxygen toxicity. This situation could be further aggravated by capillary destruction (24) and leakage into the alveolar spaces of blood components which inactivate surfactant (28).

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