Induction of Superoxide Dismutase by Oxygen in Neonatal Rat Lung*

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Neonatal rats are markedly resistant to oxygen toxicity when compared with adult animals. This study was designed to understand better some of the underlying biochemical processes in the lung which accompany oxygen exposure of the neonatal rat with the aim of developing an explanation for the oxygen tolerance. Superoxide dismutase activity was determined in neonatal rat lung tissue after either exposure to a normobaric atmosphere of 95% oxygen for 24 h or incubation of excised minced lung in an atmosphere of 100% oxygen for 5 h. Enzyme activity of this oxygen-treated tissue was compared with values obtained from air-exposed lung tissue. Hyperoxic exposure either in vivo or incubation of excised minced lung in an atmosphere of 100% oxygen for 5 h. Enzyme activity of this oxygen-treated tissue was compared with values obtained from air-exposed lung tissue. Hyperoxic exposure either in vitro or in vivo produced an increase in total lung superoxide dismutase activity. The increased activity resulting from both types of exposure conditions showed identical characteristics upon column chromatography when compared with the unstimulated activity. Cellular integrity was found to be necessary for the effect to occur in the in vitro exposure system since hyperoxic incubation of broken cell preparations did not result in enhanced superoxide dismutase activity. The increase in enzymatic activity was accounted for solely by a change in the mitochondrial manganese superoxide dismutase. The oxygen-mediated effect could not be explained by proliferative changes in either the lung cell population or the pulmonary cell mitochondrial number. Puromycin, actinomycin D, and cycloheximide used at concentrations known to inhibit protein synthesis, abolished the oxygen enhancement of superoxide dismutase activity. Incorporation of [3H]leucine into lung mitochondrial superoxide dismutase was stimulated by both in vitro and in vivo exposure to hyperoxic conditions. Furthermore, this stimulation of amino acid incorporation was inhibited by protein synthesis inhibitors in a similar manner as was the increase in enzymatic activity. All of these findings are compatible with the view that specific synthesis of mitochondrial superoxide dismutase occurs in neonatal rat lung tissue after exposure to a high concentration of oxygen. This may account in part for the resistance of neonatal rats to oxygen toxicity.

It is now known that the activity of the enzyme superoxide dismutase can be increased in prokaryotic cells as a consequence of extended exposure to high concentrations of oxygen. Fridovich and his colleagues have collected considerable evidence that microorganisms such as Escherichia coli and Streptococcus faecalis grown under hyperoxic conditions will show a 10-fold increase in enzymatic activity when compared with organisms grown anaerobically (1, 2). A concomitant tolerance to the toxic effects of hyperoxic exposure was observed in cells with increased superoxide dismutase activity. These studies were extended to the eukaryotic organism, Saccharomyces cerevisiae. Superoxide dismutase activity increased nearly 7-fold in these cells after growth in 100% oxygen.

Studies with higher animals have been performed in which superoxide dismutase activity in lung was assessed following hyperoxic exposure. A correlation between the oxygen-mediated increase in rat pulmonary superoxide dismutase activity and the resistance to the toxic effects of hyperoxia was reported by Crapo and Tierney (3). Recently Cross and his associates showed a large increase in the activity of both superoxide dismutase and other "antioxidant" enzymes in the lungs of rats exposed to 90% oxygen (4). In both of these studies, only the response of mature animals was examined. Furthermore, neither group conducted tests which would allow the identification of the underlying molecular mechanisms responsible for the oxygen effect. No significant increase in enzymatic activity in the mature animals appeared until after at least 3 days of hyperoxic exposure. Findings recently reported from this laboratory showed that whereas extended oxygen exposure was required in order to effect a measurable increase in superoxide dismutase activity in the lungs of mature animals, only 24 h of hyperoxic exposure was necessary for significant elevation of superoxide dismutase activity in neonatal rat lungs (5). In addition, a similar increase in enzymatic activity occurred if excised, minced, neonatal rat lungs were incubated in an atmosphere of 85 to 100% oxygen. The oxygen-mediated increase in activity was shown to be age-dependent over the first 3 weeks after birth. In both in vitro and in vivo exposure systems, the maximum increase occurred 10 days after birth.

Since immature animals of certain species are known to be extraordinarily resistant to oxygen toxicity (6-8) it is apparent that adaptive biochemical processes directed toward the protection of the lung at birth (9) must be present. The oxygen-provoked increase in superoxide dismutase activity in neonatal lungs previously reported by this laboratory (5) could be

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one such adaptive process. The molecular nature of this special effect has been further studied and is the subject of this report.

MATERIALS AND METHODS

Animals - The Sprague-Dawley rats utilized for these studies were bred and raised at The University of Iowa Animal Care Facility. Mature female rats were exposed to a 12-h breeding period. The onset of pregnancy was timed from the midpoint of this 12-h period. An earlier report from this laboratory showed that the maximum increase in superoxide dismutase activity due to either in vivo or in vitro oxygen exposure occurred in the lungs of neonatal rats 10 days after birth (6). Only animals of this age, therefore, were used for these studies.

Oxygen Exposure - For the experiments in which neonatal rats were exposed in vivo to hyperoxia, animals were maintained for 24 h in a controlled atmosphere chamber filled with 95% oxygen. The O₂ concentration was monitored with a Beckman model OM-11 gas analyzer and kept below 0.6%. After the exposure period, the animals were decapitated and the lungs immediately removed en bloc. The tissue was perfused with ice-cold isotonic potassium phosphate buffer, pH 7.4, blotted dry, and weighed.

In studies in which lung tissue was exposed to hyperoxia in vitro, neonatal rats were killed by decapitation, and the lungs immediately removed en bloc. The tissue was perfused with ice-cold isotonic potassium phosphate buffer, pH 7.4, blotted dry, and minced with a razor blade to a size of 1 to 2 mm³. Lung tissue was obtained from several litters of animals and was pooled prior to each experiment. Mincéd lung tissue (0.1 g per sample) was then added to incubation vessels containing 1 ml of adult rat plasma and 3 ml of isotonic potassium phosphate buffer, pH 7.4, according to our previously reported procedure (5). The tissue was incubated in either air or 100% O₂, at 37° for 5 h.

Homogenate Preparation - Potter-Elvehjem homogenates (10%, wt/v) were prepared from both the air- and the O₂-incubated tissue samples by four consecutive passes through this tissue homogenizer. Omni-Mixer homogenates were prepared from the above described homogenates using a 50-ml capacity Sorvall Omni-Mixer. Homogenization was continued for 3 min at 0-4° at a speed setting of 6.5. Low speed centrifugation was performed in a Sorvall RC-2B refrigerated centrifuge equipped with a SS34 rotor. High speed centrifugation was performed in a Beckman L-2 centrifuge equipped with a SW 56 rotor. Protein concentration was assayed by the micro-biuret method (10).

Enzyme Assays - Superoxide dismutase activity was measured by the method of McCord and Fridovich (11). The assay mixture contained 10 μM ferricytochrome c, 100 μM xanthine, 50 mM potassium phosphate, pH 7.8, 0.1 mM EDTA, 0.015 to 0.02 μM xanthine oxidase, and lung homogenates or partially purified superoxide dismutase preparations. One unit of superoxide dismutase activity was defined as the amount which inhibited the rate of reduction of ferricytochrome c by 50%. The reaction rate of a sample containing no lung tissue homogenate was measured at 550 nm in a Varian Spectrophotometer model 635D at 25° to obtain a reference rate. Increasing volumes of various lung preparations were then introduced into the incubation mixture, until the reaction rate dropped to 40 to 60% of the reference rate. The volume of sample material required was recorded and the superoxide dismutase activity calculated per ml.

Cytochrome c oxidase activity was measured by the method of Wharton and Tsagoloff (12). Succinate dehydrogenase activity was measured by the method of Slater and Bonner (13). Isocitrate dehydrogenase activity (NAD⁺ dependent) was measured in the presence of 3 mM ADP by the method of Grafflin and Ochoa (14). Glutamate dehydrogenase activity was measured by the method of Schmidt (15).

RESULTS

An earlier report from this laboratory described an increase in superoxide dismutase activity which occurred in neonatal rat lung tissue after both in vivo and in vitro exposure to 95 to 100% oxygen (6). Initially, the in vitro exposure studies were conducted on minced lung tissue. To determine whether this increase in enzymatic activity required the presence of whole lung cells, various lung tissue preparations were exposed to either air or 100% oxygen under the above described conditions. Table I shows that, as previously reported, superoxide dismutase activity increased appreciably (43%) when minced lung tissue was incubated under hyperoxic conditions. In contrast to this, however, incubation of homogenized lung tissue prepared with either a Potter-Elvehjem tissue disrupter or a Sorvall Omni-Mixer failed to produce an oxygen-mediated increase in enzymatic activity. Furthermore, incubation of either the supernatant fraction or the resuspended pellet obtained after centrifugation of each homogenate did not produce a significant increase in enzymatic activity. The small increase in activity that was observed after incubation of the 15,000 × g pellet fraction may represent activity from cells which escaped disruption. From these data, we have concluded that maintenance of cellular integrity during hyperoxic exposure is necessary in order that the increase in superoxide dismutase activity will be obtained.

Since superoxide dismutase is known to be present in two different forms in the cells of eukaryotes (16, 17), studies were conducted to ascertain whether the observed increase in activity after oxygen exposure was a result of a change in the cytoplasmic cuprozinc superoxide dismutase activity, the mitochondrial manganese enzyme activity, or both. After incubation of minced lung tissue, both a Potter-Elvehjem homogenate and an Omni-Mixer homogenate were prepared in order to determine the degree of tissue disruption that was necessary to measure the oxygen-stimulated increase in activity. As shown in Table II, the oxygen-mediated increase in activity was not observed with the homogenates, although homogenates prepared in a Potter-Elvehjem homogenizer showed an increase in superoxide dismutase activity that was significant at the 0.05 level. It was subsequently determined that the increase in superoxide dismutase activity obtained with the heterologous homogenates was attributable to the presence of homogenate released from the tissue during homogenization.

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TABLE I

| Tissue preparation | Superoxide dismutase activity | Air | 100% O₂ | Percent increase |
|--------------------|-----------------------------|-----|---------|----------------|
| Homogenate         |                            |     |         |                |
| Minced whole lung  | 0.7                        | 1.0 | 43      |
| Homogenate/Potter-Elvehjem | 0.7 | 0.7 | 0     |
| Supernatant fraction | 0.8 | 0.8 | 0     |
| Pellet             | 0.8                        | 0.9 | 12      |
| Homogenate Omni-Mixer | 0.7 | 0.7 | 0     |
| Supernatant fraction | 0.7 | 0.7 | 0     |
| Pellet             | 0.75                       | 0.8 | 7       |

a After in vitro incubation, the Potter-Elvehjem homogenates and subsequently prepared subcellular fractions were further homogenized in the Omni-Mixer before assay of the enzymatic activity.

These fractions were obtained after centrifugation of each homogenate at 15,000 × g for 15 min.

TABLE II

| Tissue treatment | Superoxide dismutase activity | Air | 100% O₂ | Percent increase |
|------------------|-----------------------------|-----|---------|----------------|
| Homogenate       |                            |     |         |                |
| Potter-Elvehjem   | 0.8                        | 0.8 | 0 (53)% |
| Supernatant fraction | 0.65 | 0.7 | 8 (10)% |
| Pellet            | 0.5                        | 0.6 | 0 (53)% |
| Homogenate Omni-Mixer | 0.7 | 1.1 | 57     |
| Supernatant fraction | 0.7 | 0.7 | 0     |
| Pellet            | 0.5                        | 0.7 | 40      |

a Percentage increase in activity after homogenization in the Omni-Mixer.

These fractions were obtained after centrifugation at 15,000 × g.
activity could be demonstrated only in the Omni-Mixer-prepared homogenate. No increase in superoxide dismutase activity above the basal level could be measured when the minced tissue was homogenized by the relatively gentle Potter-Elvehjem procedure. The oxygen-mediated effect had occurred, however, in this latter tissue preparation since further tissue disruption of the Potter-Elvehjem homogenate in the Omni-Mixer yielded preparations in which both the basal and incremental activity was seen. The resuspended pellet obtained after centrifugation of the Omni-Mixer homogenate at 15,000 \( \times g \) for 15 min (a fraction which roughly corresponds to isolated mitochondria), was the only subcellular fraction in which both the basal and the oxygen-mediated increase in enzyme activity could be detected. The resuspended pellet from the Potter-Elvehjem homogenate showed only the basal activity. However, Omni-Mixer homogenization of this pellet, as with similar treatment of the tissue, yielded a fraction in which the increase in activity could be observed. It appears, therefore, that the oxygen-enhanced enzyme activity is detected only after vigorous homogenization. Analysis of lung tissue obtained from animals exposed in vivo to either air or \( 100% \) oxygen, therefore, that the oxygen-enhanced enzyme activity is detected only after vigorous homogenization. Analysis of lung tissue obtained from animals exposed in vivo to either air or \( O_2 \), gave results similar to those described above.

The two forms of superoxide dismutase differ with respect to their sensitivity to cyanide (16). Cytoplasmic superoxide dismutase is markedly inhibited in the presence of 1 \( mM \) cyanide, whereas the mitochondrial enzyme is unaffected. By utilizing this distinguishing characteristic, we sought confirmation that only the mitochondrial superoxide dismutase was affected by hyperoxia. In Table III it can be seen that in the whole tissue homogenate as well as in the crude mitochondrial fraction, the activity increase is not diminished in the presence of cyanide. We conclude therefore that only the mitochondrial enzyme is associated with the oxygen effect.

Previous data from this laboratory reported that the oxygen-mediated increase in enzymatic activity was observed regardless of whether the specific activity was calculated in terms of lung weight, lung protein, or lung DNA content (5). These results as well as the observation that the activity of the cuprozinc enzyme is unchanged strongly suggested that oxygen-stimulated cell proliferation was not the cause of the hyperoxic effect. However, oxygen-stimulated mitochondrial proliferation could explain the results. To test this possibility, the activity of certain mitochondrial marker enzymes was determined in Omni-Mixer preparations of minced lung tissue after incubation in 100% oxygen and compared with the activity of similar preparations that were incubated in air. Neither cytochrome oxidase and succinate dehydrogenase (enzymes associated with the mitochondrial inner membrane), nor isocitrate dehydrogenase and glutamate dehydrogenase (enzymes associated with the mitochondrial matrix) change under conditions where superoxide dismutase activity is increased (Fig. 1).

To ascertain whether the increase in superoxide dismutase activity was the result of the synthesis of a new form of the mitochondrial enzyme, a comparison was made of the chromatographic characteristics of basal and oxygen-stimulated mitochondrial superoxide dismutase. After exposure to 95% oxygen or air, either in vitro or in vivo, excised lung tissue was homogenized with the Potter-Elvehjem homogenizer and the crude mitochondrial fraction prepared. After sonication, the preparation was centrifuged at 105,000 \( \times g \) for 60 min and the pellet discarded. As seen in Table IV, this procedure released both the basal and the oxygen-stimulated mitochondrial enzyme activity from the membrane. Ammonium sulfate was added to the supernatant fraction to 60% of saturation and the resultant suspension was centrifuged at 105,000 \( \times g \) for 60 min after which the pellet was discarded. Following dialysis of the supernatant fraction against 0.05 \( M \) Tris/HCl, pH 7.8, the solution containing cyanide-resistant superoxide dismutase activity was applied to a Sephadex G-100 column and the activity eluted with 0.05 \( M \) Tris/HCl. Fractons containing the cyanide-resistant superoxide dismutase activity were pooled and dialyzed overnight against 0.005 \( M \) Tris/HCl. This crude preparation was subjected to ion exchange chromatography on a column of DEAE-cellulose with an applied Tris/HCl gradient of increased ionic strength from 10 to 100 \( m\). This procedure represents a modification of that developed by Weisiger and Fridovich (16). The results depicted in Figs. 2 and 3 show that the activity eluted in a single peak at the same ionic strength regardless of whether the initial hyperoxic exposure of the pulmonary tissue occurred in vivo or in vitro. In addition,
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Table IV

| Tissue sample       | Supern oxide dismutase activity | Air   | 100% O₂ | Percent increase |
|---------------------|---------------------------------|-------|---------|-----------------|
|                     | units/ml                        |       |         |                 |
| Mitochondria (whole)| 1.7                             | 1.6   | 0       |                 |
| Mitochondria (broken)| 1.7                            | 2.1   | 24      |                 |
| Mitochondria (sonicated)| 1.6                        | 2.0   | 25      |                 |
| Supernatant         | 1.6                             | 2.1   | 32      |                 |
| (165,000 × g)       |                                 |       |         |                 |
| Resuspended         | <0.1                            | <0.1  | 0       |                 |
| (165,000 × g)       |                                 |       |         |                 |

* Units of cyanide-resistant superoxide dismutase activity per ml of mitochondrial suspension. All assayed samples contained identical volumes of material.

* These preparations were obtained by resuspension of the 15,000 × g pellet following centrifugation of homogenized (Potter-Elvehjem), previously incubated minced lung tissue of 10-day-old rats.

* The preparation was similar to that described above but subjected to additional homogenization in the Omni-Mixer before enzymatic assays were conducted.

* The preparation was similar to that described in Footnote b but subjected to sonic oscillation four times for 3 min each at a power setting of 20.

Fig. 2. Ion exchange chromatography of partially purified lung mitochondrial superoxide dismutase on DEAE-cellulose equilibrated in 0.005 M Tris/HCl buffer at pH 7.8. The conditions were identical with those described for Fig. 2, except that lung tissue was excised from 10-day-old rats, minced, and then exposed to either air or 100% oxygen in vitro as described in the text.

Fig. 3. Ion exchange chromatography of partially purified lung mitochondrial superoxide dismutase on DEAE-cellulose equilibrated in 0.005 M Tris/HCl buffer at pH 7.8. The conditions were identical with those described for Fig. 2, except that lung tissue was excised from 10-day-old rats, minced, and then exposed to either air or 100% oxygen in vitro as described in the text.

**DISCUSSION**

There is increasing evidence that both prokaryotic and eukaryotic aerobic organisms respond to hyperoxic exposure with direct and specific protein synthesis (1, 2, 20). The reported increase in superoxide dismutase activity in both Streptococcus faecalis and Escherichia coli B following growth in 100% oxygen represents the first of these observations (1). Studies show that Saccharomyces cerevisiae also responds to inhibitors of RNA-dependent protein synthesis (19). Following hyperoxic pressure, the tissue was homogenized and assayed for superoxide dismutase activity. These results were compared with data obtained from similarly treated tissue incubated in air. Actinomycin D greatly diminished the oxygen-mediated increase in superoxide dismutase activity in the in vitro system (Fig. 4). The oxygen effect was completely abolished when the incubation medium contained either puromycin or cycloheximide. A similar loss of the oxygen effect was seen when cycloheximide was administered intraperitoneally to 9-day-old rats prior to in vivo exposure to hyperoxia.

As a further test for the occurrence of oxygen-induced protein synthesis, the extent of incorporation of [3H]leucine into mitochondrial superoxide dismutase was determined. After incubation, the lung tissue was homogenized, the mitochondrial fraction recovered by centrifugation and the manganese superoxide dismutase partially purified according to the procedure described above. As seen in Figs. 5 and 6, exposure of lung tissue to 95% oxygen either in vivo or in vitro greatly enhanced the incorporation of [3H]leucine into fully purified mitochondrial superoxide dismutase. Furthermore, as expected, both puromycin and cycloheximide inhibited the incorporation of [3H]leucine into the oxygen-induced newly synthesized mitochondrial superoxide dismutase. Reference to the inset diagrams in Figs. 2 and 3 show that upon ion exchange chromatography the elution pattern of mitochondrial superoxide dismutase. Furthermore, as expected, both puromycin and cycloheximide inhibited the incorporation of [3H]leucine into the oxygen-induced newly synthesized mitochondrial superoxide dismutase. Reference to the inset diagrams in Figs. 2 and 3 show that upon ion exchange chromatography the elution pattern of mitochondrial superoxide dismutase activity and the elution pattern of the oxygen-enhanced [3H]leucine incorporation into mitochondrial protein are identical. This relationship holds regardless of whether the lung tissue was exposed to hyperoxia in vivo or whether in vitro exposure to oxygen was employed with excised minced tissue.
hyperoxia in a similar manner (20). In the yeast, both the cuprozinc and the manganesezyme increased in activity.

The results reported here clearly show that in the lung tissue of neonatal rats, a specific protein synthetic capacity is linked to the oxygen tension to which the pulmonary tissue is exposed and that a rapid oxygen-directed synthesis of the mitochondrial superoxide dismutase occurs. This response appears somewhat different than that described for adult rat lung tissue in that (a) it occurs very rapidly (within 24 h) and (b) can be reproduced in excised tissue. Both characteristics make it unlikely that the response could be associated with oxygen-induced, pulmonary reparative-proliferative changes as suggested by Cross and his associates (4).

Since the neonatal lung is known to be very resistant to the toxic effects of hyperoxia (6-8), it is possible that this rapid enzyme synthesis more nearly represents the adaptive and defensive response to hyperoxia described by Fridovich and his colleagues in the prokaryotic and simple eukaryotic systems. In their studies, enhanced intracellular levels of superoxide dismutase, as well as of catalase and peroxidase, were directly associated with protection against oxygen toxicity (1, 2, 20, 21). In higher animals such a defense mechanism would be important primarily to the lung since this organ is one of those directly exposed to the environment. Because certain animals such as rats and humans are abruptly thrust at birth from a semiaerobic environment of the uterus to the relative hyperoxia of air and, because the lung would necessarily be required to cope rapidly with such an environmental change, it is not unreasonable to propose that rapidly responding endogenous protective mechanisms should be present in neonatal lung. Available evidence indicates that of all organs of adult animals tested only the lung is capable of enhanced superoxide dismutase activity following \textit{in vivo} exposure to hyperoxia (3, 22). A similar test has not been conducted, as yet, for neonatal animals.

Although pulmonary morphologic and functional changes accompanying oxygen toxicity have been described (8, 9), the underlying molecular events are not well understood either as to the primary location or the initiating agent. It is possible that both the primary toxic agent and the enzyme inducer could be either molecular oxygen or a product of its metabolism. Our work clearly shows that one event accompanying hyperoxic exposure, that of specific enzyme induction, occurs in the mitochondria of lung tissue. We have no evidence as yet whether this protein synthesis is directed from the nuclear genome or mitochondrial DNA although present evidence favors the former location for the synthesis of mitochondrial superoxide dismutase (17).

Maintenance of viable mitochondria in the presence of potentially damaging agents such as oxygen is important for lung function. Superoxide-free radicals are known to be generated by many enzymes (11, 23-25) including those located in the mitochondria (26). In the presence of high concentrations of oxygen, the flux of oxygen free radicals generated by enzymatic reactions is elevated (27). It follows then, as has already been suggested for liver cells (26), that pulmonary mitochondrial superoxide dismutase may function to preserve the molecular integrity of the oxygen-metabolizing system against the by-products of this system. In response to hyperoxic exposure, the protein synthetic apparatus of the affected cells may be programmed to elevate the levels of this endogenous protective enzyme, superoxide dismutase, in the appropriate subcellular location. The ultimate effect could be, in concert with other such endogenous protective mechanisms, to diminish the toxic effect of hyperoxia.

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