AN EARLY MARKER OF HYPEROXIC LUNG INJURY IN THE RAT AND ITS PHARMACOLOGICAL MODULATION

M. J. O'CONNELL, S. D. SNAPE AND J. F. NUNN

SUMMARY
Of three possible early biochemical changes which were investigated in rats after hyperoxia, one was shown to be a useful marker of damage in this species. Mitochondrial oxygen uptake measured in lung homogenates has already been reported to be impaired after 24 h. With a purified mitochondrial fraction, we found significant impairment after only 3 h exposure to 100% oxygen. To our knowledge, this is the earliest significant change reported in this species. The antioxidants N-acetyl cysteine, dimethyl sulphoxide and allopurinol were found to ameliorate the injury. This suggests a possible link with the pulmonary damage and survival of rats in hyperoxia, which may be modulated also by antioxidant therapy.

KEY WORDS

Exposure of patients to high concentrations of oxygen is routine in many situations including intensive therapy, anaesthesia, radiotherapy and diving. Such exposure is known to cause serious lung injury in laboratory animals. Pleural effusion, increased lung capillary permeability and pulmonary oedema are detectable in the rat from about 48 h exposure to 100% oxygen [1-3].

There is evidence that hyperoxia increases production of both the superoxide free radical and hydrogen peroxide in isolated mitochondria in vitro [4, 5]. Antioxidant induction or therapy has been found to increase survival times [6-8] and reverse changes in lung permeability (assessed by \(^{125}\)I-albumin flux) in rats exposed to 100% oxygen [3]. However, such studies investigate events late in hyperoxic exposure and cannot represent the primary biochemical changes. The aim of the present study was to investigate possible early markers of damage in hyperoxia. This should help in defining the nature of early injury and be useful in the development of effective prophylactic measures. In addition (if the technique could be applied in patients) it would provide a basis for early diagnosis of hyperoxic lung injury.

Three studies have reported biochemical changes in the first 24 h of exposure to hyperoxia. In the first, lung homogenates from rats exposed to 100% oxygen for 24 h showed reduced uptake of oxygen (in the presence of succinate and ADP) compared with homogenates from control animals [9]. In the second, when rabbits underwent ventilation with an increased \(F_{\text{t}0_2}\) for 30 min, the lung microsomal phospholipid fatty acid composition was found to be significantly altered [10]. In the third, acid precipitable hydroxyproline was reported to be significantly increased in endobronchial washings from rats after 24 h exposure to 100% oxygen [11]. These reports led us to explore further the use of these techniques (and their derivatives) as potential early markers of hyperoxic injury. We measured oxygen uptake by purified lung mitochondrial fractions, mitochondrial and microsomal phospholipid fatty acid compositions and hydroxyproline concentrations in endobronchial washings. Significant biochemical changes within 24 h exposure were followed up by experiments to determine if these changes could be modulated by antioxidant therapy. Of the four compounds used, N-acetylcysteine and dimethylsulphoxide are predominantly hydroxyl radical scavengers. Allopurinol is also an inhibitor of xanthine oxidase, which is a source of the superoxide radical. Desferrioxamine owes much

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of its antioxidant capacity to its metal chelation properties.

MATERIALS AND METHODS

Oxygen exposures

Rats (female Sprague-Dawley, 160–200 g body weight) were exposed to 100% oxygen or air in a large Perspex container into which a conventional cage containing six animals could be placed [3]. Oxygen or air was delivered at 15 litre min⁻¹. Slight positive pressure (2–3 cm H₂O) was maintained to prevent ingress of air. Exposure was for a maximum of 60 h and the animals showed no sign of respiratory (or other) distress. A 12-h light and dark schedule was maintained and rats were allowed diet and water ad libitum.

Lung mitochondrial oxygen uptake

Groups of rats were exposed to oxygen or air for 0, 3, 24 or 48 h. Rats were then anaesthetized with halothane and killed by cervical dislocation. Lungs were removed and placed in 10 ml of ice-cold buffer (sucrose 0.225 mol litre⁻¹, potassium phosphate 10 mmol litre⁻¹ pH 7.4, magnesium chloride 5 mmol litre⁻¹ and triethanolamine 20 mmol litre⁻¹ pH 7.4) and were homogenized using a Silverson laboratory emulsifier at maximum speed for 30 s. Homogenates were centrifuged initially at 600 g for 10 min, the supernatants were transferred and centrifuged further at 10000 g for 10 min. The mitochondria-rich pellets were resuspended in 1 ml of buffer, equilibrated with air at 37 °C. Oxygen uptake rates were determined with a Clarke electrode. A background rate was recorded with 500 ul of mitochondrial fraction made up to 3.5 ml with buffer. State 3 (ADP stimulated) respiration was recorded by adding final concentrations of sodium succinate 3.3 mmol litre⁻¹ and ADP 0.3 mmol litre⁻¹. Rates of uptake of oxygen per minute were related to total lungs (lung wet weight, dry weight and protein can all change in oxygen exposure).

Lung mitochondrial and microsomal phospholipid fatty acid composition

Groups of rats were exposed to oxygen or air for 1 h or 24 h. Mitochondrial fractions were prepared as described above. Microsomal fractions were prepared by centrifuging the supernatant from the 10000 g spin at 100000 g for 1 h (at 4 °C). Both fractions were resuspended finally in 500 µl of buffer. Lipids were extracted by adding 5 ml of chloroform–methanol (2:1 by volume) containing butylated hydroxytoluene 10 mg litre⁻¹. The mixtures were shaken for 10 min and centrifuged at 1000 g for 10 min. The upper (chloroform) layer was transferred and evaporated. The extracted lipids were taken up in chloroform 250 ml. The extract was applied (50 µl in a 1-cm band) to a thin layer chromatography plate (Analtech, silica gel G, 250 µm) and developed with a mobile phase of hexane–diethyl ether–acetic acid (80:20:2 by volume). Solvent was evaporated from the plate and visualized by brief exposure to iodine vapour. Lipid classes were identified by comparisons of migrations with those of authentic standards. The phospholipid spot (origin) was transferred to a screw-cap tube for esterification. Methanolic sodium hydroxide (400 µl of 0.5 mol litre⁻¹) was added and the capped tubes were heated at 100 °C for 5 min. Boron trifluoride reagent 500 ml (Sigma) was added and the tubes heated at 100 °C for a further 2 min. The methyl esters were extracted into hexane and analysed with a Pye-Unicam 204 gas chromatograph.

Acid precipitable hydroxyproline in endobronchial washings

Groups of rats were exposed to oxygen or air for 0, 24, 48 or 60 h. At the end of the exposure rats were anaesthetized by i.p. injection of pento-barbitone 30 mg (Sagatal). The thorax was opened and a cannula was tied into the trachea. The heart and lungs were removed intact and bathed in warm physiological saline. The contents of the lungs were washed out with saline 2 ml. This was repeated twice and the three washings were pooled and centrifuged at 600 g for 10 min. The supernatants were added to concentrated hydrochloric acid (HCl) 3 ml. The mixture was centrifuged at 1500 g for 30 min. The pellet was taken up in water 2 ml, added to a further 3 ml of concentrated HCl and hydrolysed by heating at 116 °C for 16 h in a screw-cap culture tube. Hydroxyproline was assayed by oxidation to pyrrole and reaction with p-dimethylamino-benzaldehyde to form a complex quantifiable photometrically (absorption at 560 nm) [12].

Antioxidant administration

Four groups of rats were prepared for each antioxidant tested. Both antioxidant-treated and control animals were exposed to oxygen or air for 24 h. Treatment with each antioxidant was started.
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TABLE I. Oxygen uptake by mitochondrial fractions from lungs of rats exposed to 100% oxygen or air (mean (SD)) (n = 6). Significant differences after 3, 24 and 48 h exposure: *P < 0.05; **P < 0.01; ***P < 0.001

<table>
<thead>
<tr>
<th></th>
<th>0 h</th>
<th>3 h</th>
<th>24 h</th>
<th>48 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>100% oxygen</td>
<td>—</td>
<td>78.6 (15.3)</td>
<td>65.4 (9.6)</td>
<td>67.8 (18.3)</td>
</tr>
<tr>
<td>Air</td>
<td>96.0 (9.9)</td>
<td>98.4 (12.9)</td>
<td>88.2 (7.5)</td>
<td>99.6 (11.1)</td>
</tr>
</tbody>
</table>

12 h before and continued throughout exposure. N-acetylcysteine was administered at 3% (w/v) in drinking water adjusted to pH 7.0 with sodium bicarbonate. Desferrioxamine was given by i.p. injection (20 mg every 12 h), controls being injected with an equivalent volume of saline. Dimethyl sulphoxide was given at 1.5% (w/v) in drinking water. Allopurinol was given at 25 mg/100 g of powdered diet, controls having powdered diet alone. Food and water were taken normally during the first 24 h of exposure to oxygen. Mitochondrial fractions were prepared and oxygen uptake measured as described above.

Statistical analysis
Data were analysed by one-way analysis of variance and then two-sample Student’s t tests as appropriate. Variances were compared by F ratio and were not significantly different. P < 0.05 was considered significant.

RESULTS

Oxygen uptake
Oxygen uptake by the mitochondrial fraction in the presence of succinate and ADP (state 3) was significantly reduced after 3, 24 and 48 h exposure to 100% oxygen (table I). The 20% reduction at 3 h was significant at the 2% level. After 24 and 48 h the reductions were 26% and 32%, respectively.

Fatty acid composition
Phospholipid from rat lung microsomal and mitochondrial fractions was analysed for fatty acid composition. No significant differences were found between data from rats exposed to 100% oxygen (for 1 h or 24 h) and control animals (tables II and III).

Hydroxyproline
The mean acid precipitable hydroxyproline content of endobronchial washings from rats exposed to 100% oxygen was greater than that from control animals (table IV). There was a trend of increased hydroxyproline content throughout oxygen exposure. However, there was considerable scatter among the data and a statistically significant difference was not reached until 60 h.

Antioxidant therapy
Three compounds afforded significant protection of mitochondrial function in rats exposed to
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TABLE III. Fatty acid composition of lung mitochondrial phospholipid from rats exposed to 100% oxygen or air (mean (SD)) (n = 6). No significant difference between oxygen-exposed and control animals

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>1 h (100% O₂)</th>
<th>24 h (100% O₂)</th>
<th>1 h (Air)</th>
<th>24 h (Air)</th>
</tr>
</thead>
<tbody>
<tr>
<td>16:0</td>
<td>29.3 (1.9)</td>
<td>32.4 (2.0)</td>
<td>32.4 (2.5)</td>
<td>32.7 (2.6)</td>
</tr>
<tr>
<td>18:0</td>
<td>12.3 (1.7)</td>
<td>10.7 (0.4)</td>
<td>13.5 (2.2)</td>
<td>13.2 (0.2)</td>
</tr>
<tr>
<td>18:1</td>
<td>12.3 (1.5)</td>
<td>13.4 (0.6)</td>
<td>12.6 (1.1)</td>
<td>14.2 (0.4)</td>
</tr>
<tr>
<td>18:2</td>
<td>9.5 (0.6)</td>
<td>9.7 (0.8)</td>
<td>8.6 (0.6)</td>
<td>8.0 (0.3)</td>
</tr>
<tr>
<td>20:4</td>
<td>13.4 (0.8)</td>
<td>13.5 (0.1)</td>
<td>12.5 (1.0)</td>
<td>11.8 (0.6)</td>
</tr>
<tr>
<td>20:5</td>
<td>1.6 (0.7)</td>
<td>1.2 (0.2)</td>
<td>1.9 (0.7)</td>
<td>1.3 (0.3)</td>
</tr>
<tr>
<td>22:6</td>
<td>3.8 (0.5)</td>
<td>4.5 (0.4)</td>
<td>4.5 (0.8)</td>
<td>3.4 (0.2)</td>
</tr>
</tbody>
</table>

Table IV. Acid precipitable hydroxyproline concentration in endobronchial washings from rat lungs after exposure to 100% oxygen or air (mean (SD)) (n = 6). ***Significant difference from air control after 60 h exposure (P < 0.001)

<table>
<thead>
<tr>
<th></th>
<th>0 h (100% O₂)</th>
<th>24 h (100% O₂)</th>
<th>48 h (100% O₂)</th>
<th>60 h (100% O₂)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydroxyproline (μg/lungs)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Air</td>
<td>2.12 (0.71)</td>
<td>2.50 (1.31)</td>
<td>3.77 (0.71)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.47 (0.43)</td>
<td>1.32 (0.69)</td>
<td>1.43 (0.55)</td>
<td></td>
</tr>
</tbody>
</table>

Table V. Effects of antioxidants on oxygen uptake by mitochondrial fractions from lungs of rats exposed to 100% oxygen or air for 24 h (mean (SD)) (n = 6). Significant difference from oxygen-exposed controls : *P < 0.05, **P < 0.01

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Antioxidant treated</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>100% O₂</td>
<td>Air</td>
</tr>
<tr>
<td>N-Acetylcysteine</td>
<td>91.2 (13.8)*</td>
<td>115.5 (16.8)</td>
</tr>
<tr>
<td>Desferrioxamine</td>
<td>64.2 (4.7)</td>
<td>102.9 (15.9)</td>
</tr>
<tr>
<td>Dimethyl sulphone</td>
<td>85.5 (9.3)**</td>
<td>115.2 (4.3)</td>
</tr>
<tr>
<td>Allopurinol</td>
<td>87.3 (5.1)*</td>
<td>115.2 (24.7)</td>
</tr>
</tbody>
</table>

100% oxygen (table V). At the doses given, N-acetylcysteine achieved 54% protection, dimethyl sulphoxide 50% and allopurinol 38%; desferrioxamine gave no protection. Despite these various degrees of protection, all oxygen-exposed and antioxidant-treated groups had mitochondrial oxygen uptake rates significantly less than those in air breathing controls.

**DISCUSSION**

Currie, Pratt and Sanders [9] reported that state 3 oxygen uptake in lung homogenates was significantly reduced after rats had been exposed to 100% oxygen for 24 h. Uptake was reported to be further reduced at 48 h, and then stabilized at this lower value for up to 7 days exposure. Using a mitochondrial fraction, we have found a significant change as early as 3 h after the start of hyperoxic exposure. The rate of uptake appears to stabilize a little earlier (in our experiments) than was reported by Currie's group, and it is possible that these authors did not achieve 100% oxygen in their exposures. Indeed, the fact that their rats survived for up to 7 days suggests that the concentration of oxygen was substantially less than 100%, in which rats do not usually survive beyond the 4th day of exposure.
Bassett and colleagues [13, 14] have studied pyruvate and glucose metabolism in isolated perfused lungs from oxygen-exposed rats and have suggested that the mitochondrial pyruvate dehydrogenase complex is a likely site of inhibition. It has long been proposed that the initial biochemical event in oxygen toxicity is the generation of oxygen-derived free radicals [15]. Clearly, mitochondria are a likely site for such reactions. Leakage of single electrons from the respiratory chain to oxygen is likely to be increased in hyperoxia. Mitochondrial enzymes are, therefore, likely targets for free radical reactions, as free radical species are often highly reactive and therefore react in the immediate locality of their formation.

Casals and colleagues [10] have reported various changes in the microsomes from lungs of rabbits which had undergone ventilation with high oxygen concentrations for only 30 min. Among the most striking of these were changes in fatty acid composition. We could find no changes in microsomal or mitochondrial fatty acid composition in lungs of rats exposed to 100% oxygen. Clearly, the two sets of experiments are not directly comparable, as there is a difference in species and experimental procedure. We can only conclude that changes in fatty acid composition are unlikely to be a useful marker of hyperoxic injury in the rat.

There is disagreement in the existing literature regarding hydroxyproline in endobronchial washings. Valimaki and Niinikoski [11] reported a very striking increase after 24 h exposure to 90–95% oxygen, while Riley and colleagues [16] detected no increase before 2.8 days. Our data are similar to those of the latter group, in that no significant increase could be detected before 60 h. Valimaki and Niinikoski [11] published in 1973 and to our knowledge their finding has not been confirmed. It would seem that endobronchial hydroxyproline is not a reliable marker of early damage in hyperoxia.

A reported early marker of oxidative lung injury, which has not been considered in the present study, is the reduction of serotonin uptake by lungs [17]. However, it has been suggested that this is secondary to the impairment of mitochondrial function [14], as such uptake requires mitochondrial energy production [18]. It would be interesting to make a careful comparison of the time courses of these two changes.

To our knowledge, this paper contains the first study of antioxidant modulation of an early marker of lung injury in hyperoxia. Up to 54% protection of mitochondrial function was achieved. Further work is required to determine if other compounds, routes of administration, doses or combinations of drugs could improve on this finding. The negative result with desferrioxamine is difficult to interpret, as it may simply indicate that the compound failed to reach the appropriate site for its action. Clearly, the positive findings with three antioxidants are consistent with a role for free radicals in the early stage of hyperoxia-dependent impairment of mitochondrial function. However, it must be stressed that they do not prove such a role, as such compounds may have various modes of action. Whatever the mechanism of protection, the findings are suggestive of a possible link between the impairment of mitochondrial function and the long term survival of rats in hyperoxia, which is also responsive to antioxidant therapy. N-Acetylcysteine and other thiols are known to be effective in this role [7], as are catalase and superoxide dismutase [8]. N-Acetylcysteine protected also against increased lung capillary permeability [3]. Dimethyl sulphoxide has shown significant protection against the appearance of pleural effusion [3]. Any link between mitochondrial function and later damage may not be causative, since both phenomena could derive from some other event. Nonetheless, the very early occurrence of the reduction in uptake of oxygen (within the first 3 h of hyperoxia and long before any overt signs of damage) suggests that it is close to the primary events in hyperoxic lung injury.

REFERENCES

5. Turrens JF, Freeman BA, Crapo JD. Hyperoxia increases


