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PROLONGED SURVIVAL AFTER PARAQUAT

ROLE OF THE LUNG ANTIOXIDANT ENZYME SYSTEMS

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Abstract—Treatment of O_2-exposed adult rats with low doses of bacterial endotoxin results in increased lung antioxidant enzyme activity and a marked protective effect against pulmonary O_2 toxicity. Since paraquat-induced lung damage is believed to be mediated by an O_2 free-radical mechanism, animals with increased lung antioxidant enzyme levels should better tolerate paraquat exposure. We therefore pre-exposed endotoxin-treated rats to hyperoxia for 48 hr and then administered a lethal dose (40 mg/kg) of paraquat to these animals and to non-O_2-exposed rats. The O_2-pre-exposed rats with elevated lung superoxide dismutase, catalase and glutathione peroxidase enzyme levels exhibited survival times after paraquat administration (LT_{50} = 7.25 days) that were prolonged more than 3-fold compared to the control, non-O_2-pre-exposed rats that had received the same dose of paraquat (LT_{50} = 2.0 days). This animal model with increased biochemical defenses against potential hyper-oxidant toxicity may be of use in studying other toxic agents that produce lung damage through an O_2 free-radical mechanism.

The herbicide paraquat (methyl viologen, 1,1'-dimethyl-4,4'-bypyridylium dichloride) has been responsible for at least several hundred deaths after accidental or intentional ingestion or injection [1, 2]. The primary cause of death is respiratory failure due to diffuse lung injury [1–4]. The toxic effect of paraquat on the lung is believed to be mediated by cytotoxic free-radicals of oxygen that are produced intracellularly during cyclic oxidation and reduction of paraquat by the metabolic processes of the cell [4, 6]. Exposure to high concentrations of O_2 also results in increased O_2-free-radical production [7, 8], and treatment with hyperoxia markedly accelerates paraquat lung toxicity [3, 9].

We have shown previously that treatment of animals with small doses of bacterial lipopolysaccharide (endotoxin) provides a marked protective effect against hyperoxic-induced lung damage and lethality [10–13]. Endotoxin appears to act by stimulating the induction of endogenous antioxidant enzyme activities in the lung—superoxide dismutase (SOD),† catalase (CAT) and glutathione peroxidase (GP) [10, 13]. These enzymes serve to detoxify cytotoxic O_2 free-radicals like the superoxide anion (O_2^-) and also to detoxify lipid peroxides formed by the interaction of reactive O_2 species with unsaturated lipids in the cell.

If excess O_2 free-radical generation is indeed responsible for paraquat-induced lung injury, then increased levels of pulmonary SOD, CAT and GP should provide a protective effect against paraquat toxicity. By pre-exposing endotoxin-treated rats to > 95% O_2 for 48 hr prior to paraquat administration, we were able to test whether animals with elevated levels of these potentially protective lung enzymes would better tolerate a toxic challenge to the lung with this herbicide.

METHODS AND MATERIALS

Adult male Sprague–Dawley rats (225–250 g) were used for these studies. The rats were maintained on standard laboratory chow and water *ad lib.* in the Animal Care Facility at the Miami Veterans Administration Hospital. The experimental animals were overtly free of any respiratory infection.

Each experimental rat was injected i.p. with a single 500 μg/kg dose of bacterial endotoxin (*Salmonella typhimurium* lipopolysaccharide, Sigma Chemical Co., St. Louis, MO) just prior to being placed in a hyperoxic exposure chamber. Control rats placed in the same chamber were injected i.p. with equivolume saline. The endotoxin-treated and control rats were then exposed to 96–98% O_2 continuously for a 48 hr period. Chamber O_2 levels and CO_2 levels (< 0.5%) were monitored frequently (Beckman OM-11 and LB-2 Medical Gas Analyzers, Beckman Instruments, Schiller Park, IL), as were chamber temperature (24–26°) and humidity (50–70%) (in-chamber thermometers and hygrometers).

Following the 48-hr exposure to 96–98% O_2, the animals were maintained in room air for 24 hr after which four of the animals from each group, together with four air controls not exposed to hyperoxia, were killed for lung enzyme assays and lung edema assessment. (Biochemical analyses are described below).

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† Abbreviations: SOD, superoxide dismutase; CAT, catalase; GP, glutathione peroxidase; and G-6-PD, glucose-6-phosphate dehydrogenase.

The remaining animals from the two O_2-exposed groups, plus rats unexposed to O_2, then received a lethal (40 mg/kg) i.p. dose of paraquat (methyl viologen, Sigma Chemical Co.) dissolved in saline. The comparative survival rates for these three treatment groups were monitored several times daily for 10 days post-injection. Statistical analysis of the comparative survival rates was done using Fisher's exact non-parametric testing [14]; LT_{50} values (time post-injection when 50 per cent of the animals had died) were compared by the graphic method of Litchfield and Wilcoxon [15]. A P value of < 0.05 was used to establish statistical significance.

Biochemical analyses. The animals killed for lung enzyme analysis and edema assessment were killed by exsanguination after the induction of anesthesia with i.p. pentobarbital. The lungs to be used for biochemical analyses were immediately perfused *in situ* via the pulmonary artery with cold isotonic phosphate buffer (0.1 M potassium phosphate–0.15 M potassium chloride, pH 7.4). The lungs were then dissected free of other thoracic tissue, blotted gently, weighed, and homogenized in cold hypotonic phosphate buffer (0.005 M potassium phosphate, pH 7.8) (10:1, volume:weight) in a Brinkman Polytron (Brinkman Instruments, Westbury, NY). Lung superoxide dismutase activity (1 unit inhibits 50 per cent of a standard reference reaction) was determined on the fresh homogenates using the ferricytochrome *c* assay [16]; lung catalase (1 I.U. reduces 1 μmole H_2O_2 per min) [17], glutathione peroxidase (μmoles NADPH oxidized per min per lung) [18], and glucose-6-PO_4 dehydrogenase (μmoles NADP reduced per min per lung) [19] activities were measured on the 15,000 *g* supernatant fluid, stored frozen overnight. These analyses involve standard spectrophotometric assays used in our laboratory for several years. The protein [20] and DNA [21] contents of the homogenates were determined using crystallized bovine serum albumin and purified calf thymus DNA (Sigma Chemical Co.) as standards for these assays.

Lung enzyme activities were compared for statistically significant differences (at $P < 0.05$ level) by Student's group *t*-test [14].

For lung edema assessment, non-perfused lungs from exsanguinated animals were treated as follows. The left lungs were ligated and weighed before and after drying to a constant weight in a 60° oven (lung wet wt/dry wt ratio); the right lungs were inflated with 10% buffered formalin solution via tracheal cannulae at a constant pressure of 20 cm H_2O. After fixation in the inflated state for 24–48 hr, each lobe was processed for hematoxylin and eosin staining. Coded slides were then examined by light microscopy.

RESULTS

Figure 1 shows the lung antioxidant enzyme levels in the two groups of animals pre-exposed to 96–98% O_2 for 48 hr (assayed just prior to the time of paraquat administration). The activities of SOD (+ 48 per cent), CAT (+ 38 per cent) and GP (+ 27 per cent) in the lung were all significantly elevated in the endotoxin-treated, O_2-pre-exposed rats compared to air control lung enzyme levels. In contrast, no significant elevations in lung enzyme levels occurred in the saline-treated O_2-pre-exposed animals (Fig. 1). Although the absolute values differed slightly, this same pattern of lung enzyme response to O_2 was observed whether activity was expressed per whole lung as shown, per g lung, per mg protein, or per

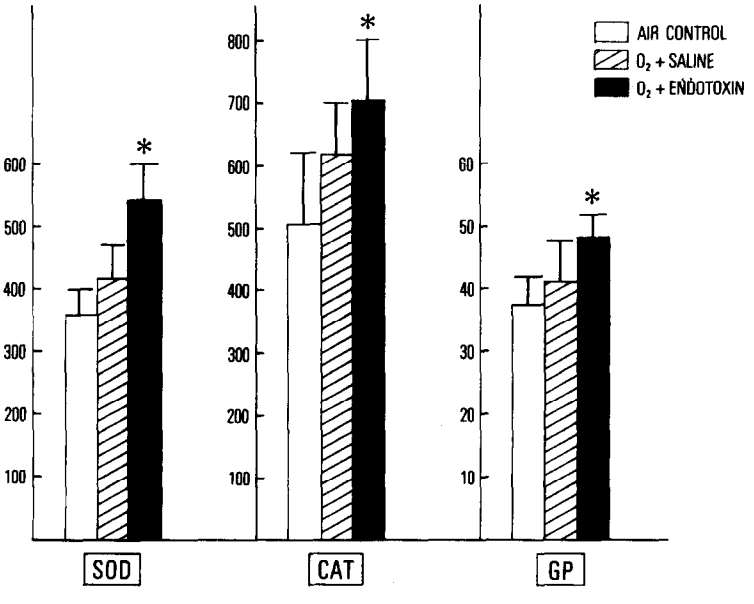

Fig. 1. Lung antioxidant enzyme activities after a 48-hr exposure to 96–98% O_2. Rats received a single injection of saline or endotoxin (500 μg/kg) at the start of O_2 exposure. The results are graphed as total activity units per lung. Values are the means ± S.D. from two experiments; there were four adult rats per group per experiment. Asterisks indicate a P value < 0.05 versus the air control enzyme value. Abbreviations: SOD, superoxide dismutase; CAT, catalase; and GP, glutathione peroxidase.

Table 1. Results of pre-exposure to O_2 for 48 hr

Treatment group*	Lung wet wt/dry wt	Lung histology
Air control	4.88 ± 0.12†	Normal
O_2 + saline	5.63 ± 0.53‡	Interstitial, peribronchial, perivascular edema
O_2 + endotoxin	5.01 ± 0.30	Normal

* Rats received a single dose of endotoxin (500 μg/kg) or saline i.p. just prior to exposure to 96–98% O_2 for 48 hr (N = 10 per treatment group; results = mean + 1 S.D.).

† $P < 0.001$ vs O_2 + saline group; not significantly different vs O_2 + endotoxin group, at $P < 0.05$ level.

‡ $P < 0.001$ vs air control and $P < 0.01$ vs O_2 + endotoxin group.

mg DNA—e.g. per DNA, changes observed were SOD (+25 per cent*, O_2 + endotoxin; and + 2 per cent, O_2 + saline group); CAT (+ 17 per cent* and + 3 per cent respectively); and GP (+ 13 per cent and + 1 per cent respectively).

The information in Table 1 indicates that hyperoxia for 48 hr did not result in evidence of pulmonary edema in the endotoxin-treated rats, as assessed histologically and by lung weight measurements. Pulmonary edema was present in the 48 hr O_2-exposed rats that had not been treated with endotoxin (Table 1).

Figure 2 illustrates the daily survival rates (composite of four experiments) after a 40 mg/kg dose of paraquat to the rats of two O_2-pre-exposed groups and to rats of a third group that had not been pre-exposed to hyperoxia. The O_2 + endotoxin-pre-exposed group had significantly higher survival rates compared to air controls on each of the first 7 days after paraquat administration. Of these animals, 53 per cent were still alive 1 week after receiving paraquat, compared to 23 per cent of the paraquat-only air controls and 33 per cent of the O_2 + saline pre-exposed groups. At day 10 after the paraquat injection, survival rates of the three groups were not statistically different (30, 25 and 16 per cent, respectively, for the O_2 + endotoxin group, O_2 + saline group and paraquat-only group).

Table 2 shows the calculated LT_{50} values for the three treatment groups, with their 95 per cent confidence intervals. The LT_{50} value for the O_2-endotoxin group (7.25 days) represents approximately a 3-fold increase over the LT_{50} values for the O_2–saline (2.5 days) and the paraquat-only group (2.0 days).

The results from a single experiment in which rats were killed 48 hr after paraquat administration to evaluate comparative lung changes and lung antioxidant enzyme levels are shown in Table 3. In addition to significant elevations in SOD, CAT, GP and G-6-PD enzyme levels, the O_2 + endotoxin treated groups showed only minimal evidence for lung edema at 48 hr after paraquat challenge. In contrast, no significant increases in lung antioxidant enzyme levels were seen in the group of rats receiving paraquat alone, and the lung dry wt/wet wt ratio was increased significantly 48 hr post-paraquat. Histological assessment of coded slides from these animals revealed prominent interstitial, peribronchiolar, and perivascular edema and scattered foci of intra-alveolar fluid accumulation in the rats that had received paraquat only. Scattered foci with intra-alveolar hemorrhage were also seen in some of the lung sections from this group. The O_2–endotoxin pre-treated groups showed interstitial edema and perivascular–peribronchiolar edema in some sections, but no evidence of intra-alveolar hemorrhage or fluid accumulation in any of the lung samples at 48 hr after paraquat administration.

* $P < 0.05$ versus air control enzyme activity.

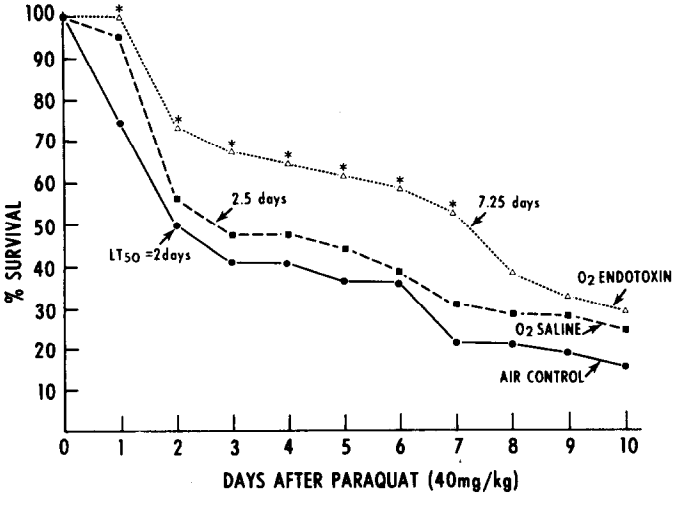

Fig. 2. Daily survival rates after a 40 mg/kg dose of paraquat. The O_2–endotoxin group had been treated with endotoxin and exposed to hyperoxia for 48 hr prior to receiving paraquat; the O_2–saline group received saline during a similar pre-exposure period in O_2; the air control group had no hyperoxic pre-exposure before paraquat. Results are composite survival totals from three to four separate experiments with ten to twelve adult rats per group per experiment. Asterisks indicate a P value < 0.05 versus the air control group.

Table 2. Lethal Time $_{50}$ values for adult rats treated with paraquat (40 mg/kg, i.p.)

Treatment group	LT_{50} (95% Confidence limits) (days)	Potency ratio*
Control	2.0 (1.09–3.33)	1.0
O_2 pre-exposure (saline)	2.5 (0.95–4.36)	1.14
O_2 pre-exposure (endotoxin)	7.3 (4.51–12.76)†	3.43†

* Potency ratio = ratio of LT_{50} values for treatment group/contol group.
† $P < 0.05$ versus other treatment groups (Litchfield–Wilcoxon test).

DISCUSSION

The pulmonary pathology seen in man and in experimental animals following paraquat ingestion or injection has been described in numerous clinical [3, 4, 22, 23] and laboratory reports [2, 9, 24–27]. The toxic mechanism of paraquat in the lung has been likened to that of hyperoxia, whose toxic action has been linked to excess O_2 free-radical production [4–8]. If this proposed mechanism for the toxic action of paraquat is a valid one, then the endogenous antioxidant defense systems of the lung can be expected to play an important role in helping to control the extent or rapidity of paraquat–O_2 free-radical cytotoxicity. Increased activities of the antioxidant enzymes SOD, CAT and GP have been shown repeatedly to be associated with protection of the lung from hyperoxic-induced lung damage and lethality [28–32]. Conversely, decreased activities of SOD, GP or selenium (GP being a seleno-enzyme) or of the lipid-phase antioxidant vitamin E have been associated with increased sensitivity to both hyperoxic- and paraquat-induced pulmonary damage and lethality [5, 30, 32, 33]. Others have suggested that the primary toxic action of paraquat is related specifically to its oxidizing (and depleting) effect on lung NADPH with resultant disruption of lung synthetic capacities and antioxidant functions [see Ref. 27 for review]. The marked increase (>200%) in G-6-PD activity in the O_2-endotoxin pre-treated rats after paraquat administration (Table 3) may then contribute importantly to the prolonged survival of this group of animals.

Previous investigators have attempted to prevent toxic lung injury due to paraquat by exogenous augmentation of the antioxidant defenses of the organism. The administration of exogenous SOD either by repeat parenteral injections or by constant infusion has been used both experimentally and clinically for this purpose but without consistent results [1, 2, 34–39]. In the present studies we made use of an earlier observation that treatment of adult rats with endotoxin during exposure to high O_2 consistently results in increases in endogenous lung antioxidant enzyme levels [10, 13]. By pre-exposing our rats to hyperoxia (and administering endotoxin at the time of O_2 exposure), the rats developed elevated lung cell levels of SOD, CAT and GP. Subsequently, when these animals with augmented antioxidant defenses were challenged with a lethal dose of paraquat, they exhibited a 3-fold prolongation of average survival time (LT_{50} = 7.25 days) compared to the survival (LT_{50} = 2.0 days) of non-pre-exposed rats. That the pre-exposure to O_2 for 48 hr was not in and of itself responsible for this relative protection from paraquat toxicity is indicated by the lack of prolonged survival post-paraquat injection in the third group of animals tested—the rats that were pre-exposed to O_2 without endotoxin treatment. These animals had no significant elevations in their enzyme activities after O_2 exposure, and they tolerated paraquat no better than the non-O_2-exposed group of rats (LT_{50} for this third group was 2.5 days).

The explanation for the incompleteness of the protection we observed (delayed histological changes and lethality) in our O_2–endotoxin pre-treated animals with increased antioxidant enzyme activities is not known. However, we can propose several possible explanations: (1) the augmented enzyme activity was still insufficient to detoxify the excessive quantities of O_2 free radicals and their products produced by paraquat; (2) the enzyme increases occurred predominately in only specific populations of lung cells (type II? endothelial cells?) and there-

Table 3. Lung antioxidant enzyme levels and pulmonary edema 48 hr after paraquat administration

Treatment group*	SOD†	CAT†	GP†	G-6-PD†	Lung wet wt/dry wt
Air control	488 ± 66	10,667 ± 665	60.2 ± 4.1	8.4 ± 2.8	4.87 ± 0.19
Control + paraquat	498 ± 85	9,323 ± 2,663	58.8 ± 10.5	11.8 ± 3.5	5.95 ± 0.83‡
O_2–endotoxin + paraquat	722 ± 142‡	12,810 ± 1,706‡	85.0 ± 16.6‡	25.5 ± 8.9‡	5.25 ± 0.37

* O_2–endotoxin animals received a single dose of endotoxin (500 μg/kg) prior to a 48-hr exposure to 96–98% O_2 before the rats in this group and in the control + paraquat group were each given a 40 mg/kg i.p., dose of paraquat. Five rats from each group were killed 48 hr post-paraquat administration for enzyme and edema measurements.
† SOD, superoxide dismutase (units/lung); CAT, catalase (International Units/lung); GP, glutathione peroxidase (μmoles NADPH oxidized·min^{-1}·lung^{-1}); G-6-PD, glucose-6-PO_4-dehydrogenase (μmoles NADP reduced·min^{-1}·lung^{-1}); values are means ± 1 S.D.
‡ $P < 0.05$ compared to air control group value.

fore only these cells were maximally protected from paraquat-oxidant stress; or (3) the antioxidant enzymes are just one part of the total cellular defense system required for complete protection from severe oxidant challenges. Of these propositions, the first is unfortunately not yet directly testable with current methodologies; the second could perhaps be elucidated in monotypic lung cell cultures (enzyme changes with hyperoxia have indeed been measured already in type II and alveolar macrophages *in vitro* [40, 41]); and the third possible explanation has already been alluded to above in the discussion of the role of NADPH and G-6-PD in paraquat toxicity, and it is considered in greater detail in Ref. 27.

The model used in this study is in some ways comparable to an animal model reported a few years ago by Bus *et al.* [42] in which rats pre-exposed to 85% O_2 for 7 days were found to have increased lung SOD activity and a nearly 2-fold prolongation of survival after administration of a lethal dose of paraquat. The important difference, however, in the two animal models with increased antioxidant enzyme levels is that after a 7-day, 85% O_2 pre-exposure regimen the lung is extensively altered, with marked evidence of pulmonary O_2 toxicity and abnormal epithelial, interstitial and endothelial cell populations [43, 44]. In contrast, the O_2-exposed endotoxin-treated animal model employed in the present study does not produce structural lung changes that are very different from the normal state ([10–12] and Table 1); only a 48-hr O_2 pre-exposure period rather than a 5- to 7-day exposure period is required to induce protective enzyme changes in the lung, and the prolongation of survival after paraquat challenge in this present model is greater than has been observed with any other manipulations (including exogenous SOD administration) reported previously. Our animal model with increased endogenous biochemical defenses against hyper-oxidant damage may be of use in investigating a variety of other pulmonary toxins whose basic mechanism of toxicity may depend on O_2 free-radical production (i.e. irradiation of the lung,* exposure to other oxidizing inhalation agents, and effects of chemotherapeutic agents like bleomycin and adriamycin).

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