SUPEROXIDE- AND SINGLET OXYGEN-CATALYZED LIPID PEROXIDATION AS A POSSIBLE MECHANISM FOR PARAQUAT (METHYL VIOLOGEN) TOXICITY[1]

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Received April 18, 1974

SUMMARY

Paraquat was reduced by mouse lung microsomes when incubated anaerobically with NADPH. The reaction was inhibited by the addition of antibody to rat liver NADPH-cytochrome c reductase. In the presence of NADPH and NADPH-cytochrome c reductase, paraquat increased the in vitro peroxidation of rat liver microsomal lipid. The peroxidation was inhibited by superoxide dismutase and the singlet oxygen trapping agent 1,3-diphenylisobenzofuran. It is suggested that paraquat toxicity may be mediated through the transfer of a single electron from reduced paraquat to oxygen and thus form superoxide ion. Singlet oxygen may form from superoxide and subsequently react with lipids to form fatty acid hydroperoxides.

INTRODUCTION

Paraquat (methyl viologen), 1,1-dimethyl-4,4'-bipyridylium dichloride, is a broad spectrum herbicide effective against broad leaf weeds and grasses. Paraquat is highly toxic to mammalian species and results in the formation of delayed pulmonary lesions characterized by progressive interstitial fibrosis which is associated with a high degree of lethality both in animals and man (1-3). The herbicidal activity of paraquat has been shown to require oxygen (4). Plant leaves incubated in paraquat solutions accumulated malondialdehyde, an indicator of lipid peroxidation (5). The toxicity of paraquat in mammals was also linked to oxygen by Fisher et al. (6), who demonstrated that rats acutely treated with paraquat were sensitized to the development of oxygen toxicity.

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[1]This study was supported in part by NIH Grants ES 00560 and GM 01761, NSF Grant GB 40711, and by the Michigan Lung Association.

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This communication presents *in vitro* evidence that the mammalian toxicity of paraquat may be a consequence of cellular lipid peroxidation. The peroxidation may be mediated through the single electron reduction of paraquat, catalyzed by NADPH-cytochrome c reductase, with the subsequent transfer of the electron from reduced paraquat to molecular oxygen to form superoxide anion. Superoxide may non-enzymatically dismutate to form singlet oxygen which will react with unsaturated fatty acids to form fatty acid hydroperoxides (7).

METHODS

Microsomes were isolated from the lungs of female Swiss-Webster mice as described by Gram (8). NADPH-Cytochrome c reductase was solubilized and purified from rat liver microsomes by the method of Pederson *et al.* (9). IgG containing antibody to NADPH-Cytochrome c reductase was prepared from the serum of rabbits immunized with purified rat liver microsomal NADPH-cytochrome c reductase by the method of Pederson *et al.* (9). Total rat liver microsomal lipids were extracted anaerobically by the method of Folch *et al.* (10) and liposomes prepared by anaerobic sonication as previously described (9). 1,3-Diphenylisobenzofuran (Aldrich Chemical Co.) was added as a suspension by co-sonication in the liposome preparation. Cytochrome c (beef heart, type IV), NADPH, and paraquat dichloride (methyl viologen) were purchased from Sigma Chemical Co. Superoxide dismutase was purified from bovine erythrocytes by the method of McCord and Fridovich (11) and protein assayed by the method of Lowry (12). *In vitro* lipid peroxidation was determined as previously described (9), using the thiobarbituric acid assay to measure the formation of malondialdehyde (13). Statistical analyses were by analysis of variance, completely random design, with mean differences detected by the least significant difference test (14). The level of significance was chosen as $p < 0.05$.

RESULTS

Mouse lung microsomes catalyzed the oxidation of 6 nmoles of NADPH per min per mg protein. In the presence of 1 mM paraquat, this value increased to 62

nmoles per min per mg protein. Under anaerobic conditions, paraquat reduction rates (measured at 395 nm) increased linearly with increasing paraquat concentrations, with maximal reduction rate reached at 1.25 mM paraquat (Fig.

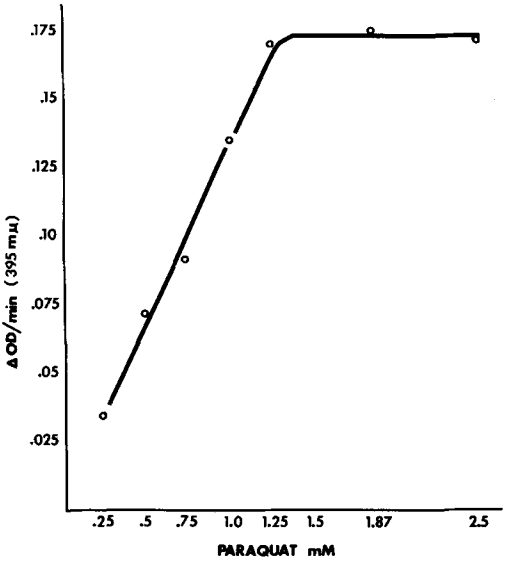

Fig. 1. Anaerobic reduction of paraquat by mouse lung microsomes. Incubation mixtures contained 60 μg/ml microsomal protein, 1 x 10^{-4} M NADPH and paraquat buffered in pH 7.5, 0.15 M potassium phosphate at 25°C. Incubation volume was 2.0 ml. Changes in optical density were recorded at 395 nm in a Coleman model 124 spectrophotometer.

1). Antibody prepared against rat liver microsomal NADPH-cytochrome c reductase inhibited the reduction of paraquat catalyzed by mouse lung microsomes (Fig. 2) with a maximal inhibition of 67%. No inhibition was observed upon incubation with pre-immune serum.

Incubation of paraquat with NADPH-cytochrome c reductase, NADPH, and microsomal lipid significantly increased malondialdehyde formation compared to the no paraquat basal rate (Table 1). Both superoxide dismutase and 1,3-diphenylisobenzofuran, a singlet oxygen trapping agent (15), showed a concentration related inhibition of paraquat-induced lipid peroxidation (Table 2). A combination of superoxide dismutase and 1,3-diphenylisobenzofuran give a

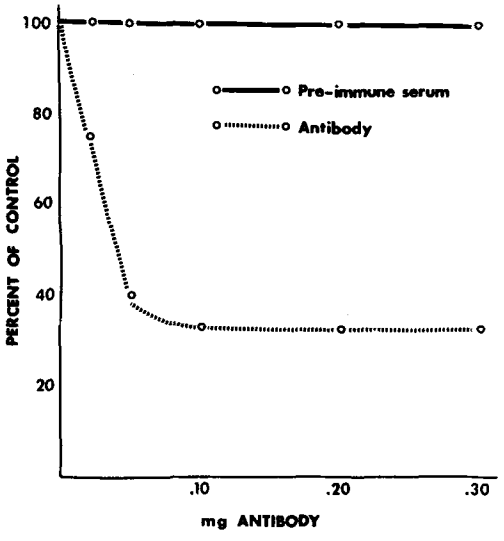

Fig. 2. Inhibition of the anaerobic reduction of paraquat by mouse lung microsomes by antibody (IgG) to rat liver NADPH-cytochrome c reductase. Incubation conditions were identical to those in Fig. 1 with the addition of either antibody or pre-immune serum and a paraquat concentration of 2.5 mM.

greater inhibition of paraquat-induced lipid peroxidation than either agent used alone.

DISCUSSION

This investigation presents evidence that paraquat-induced pulmonary lesions in mammalian species may involve peroxidation of cellular unsaturated lipids. In vitro incubation of paraquat with purified microsomal lipid and NADPH-cytochrome c reductase increased malondialdehyde generation in a concentration-dependent manner. A possible sequence of reactions to explain the stimulation of lipid peroxidation is suggested from the data.

The first reaction in the sequence was indicated in a report of Gage (16) that rat liver microsomes were capable of catalyzing a single electron reduction of paraquat which was NADPH dependent. Evidence that the reduced form was involved in paraquat toxicity was not presented. However, the present data shows that paraquat can be reduced by lung tissue, the site of the specific lesion. Inhibition of microsomal paraquat reduction by antibody to rat liver

TABLE 1. Paraquat-induced lipid peroxidation. Incubation mixtures contained 0.25 M NaCl, 2.0 mM ADP, 0.12 mM $Fe(NH_4)_2(SO_4)_2$, 0.5 μmoles/ml lipid phosphorus, 0.2 mM NADPH, 60 μg/ml liver microsomal NADPH-cytochrome c reductase, and paraquat in 0.25 M TRIS buffer, pH 6.8. Incubations were conducted at 37°C in an oscillating Dubnoff incubator under air. Total incubation volume was 5.0 ml.

Paraquat concentration per incubation mixture	Malondialdehyde formed[1] (nanomoles/min/ml)	% Increase in malondialdehyde formed
0	0.37 ± .01	0
10^{-6} M	0.43 ± .03	16.2
10^{-5} M	0.60 ± .02[2]	62.2
10^{-4} M	1.21 ± .09[2]	227.0

[1]Mean ± S.E. of 3 determinations.
[2]Significantly different from no paraquat, $p < .05$.

TABLE 2. Inhibition of paraquat-induced lipid peroxidation by superoxide dismutase and 1,3-diphenylisobenzofuran. Incubation conditions were identical to those given in Table 1 except as described below.

Incubation	Malondialdehyde formed (nanomoles/min/ml)]	% of 10^{-4} M paraquat incubation
10^{-4} M Paraquat	0.84 ± .09	---
Plus superoxide dismutase:		
20 μM	0.60 ± .06[2]	71.4
60 μM	0.28 ± .04[2]	33.3
Plus 1,3-diphenylisobenzofuran:		
2.0 μM	0.72 ± .02	85.7
10.0 μM	0.45 ± .03[2]	53.6
Plus superoxide dismutase (20 μM) and 1,3-diphenylisobenzofuran (10.0 μM)	0.11 ± .02[2,3]	13.1

[1]Mean ± S.E. of 3 determinations corrected for no paraquat control.
[2]Significantly different from 10^{-4} M paraquat, $p < .05$.
[3]Not significantly different from basal rate, $p < .05$.

NADPH-cytochrome c reductase suggests that NADPH-cytochrome c reductase may catalyze the production of reduced paraquat in the lung, with NADPH serving as an electron donor. Pulse radiolysis studies have shown that reduced paraquat

reacts with oxygen with the transfer of a single electron to form superoxide ion (17). The inhibition of paraquat-induced lipid peroxidation by superoxide dismutase provides further evidence that superoxide production may be stimulated in the presence of paraquat. Pederson and Aust (18), utilizing superoxide dismutase and the singlet oxygen trapping agent, 1,3-diphenylisobenzofuran, concluded that xanthine oxidase-induced lipid peroxidation was also mediated by the production of superoxide ion which was followed by the generation of singlet oxygen and subsequent reaction with unsaturated fatty acids to form fatty acid hydroperoxides. Evidence for a similar mechanism for paraquat-induced peroxidation is indicated by the inhibition of peroxidation by superoxide dismutase and 1,3-diphenylisobenzofuran when used alone and their synergistic effect when used in combination.

The results of this investigation offer an explanation for the paraquat enhanced oxygen toxicity observed by Fisher et al. (6). Under increased oxygen tension, higher in vivo levels of the highly toxic superoxide anion may be produced in the presence of paraquat. Furthermore, if oxygen is involved in the toxicity of paraquat, it may account for the specific lung damage caused by paraquat since this organ has the highest oxygen tension in the body. The involvement of lipid peroxidation in paraquat toxicity suggests study of the possible interactions of paraquat with such lipid peroxidation protecting agents as vitamin E and selenium.

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