# An Enzymatic Protective Mechanism against Lipid Peroxidation Damage to Lungs of Ozone-Exposed Rats

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## ABSTRACT

The effects of whole animal exposure to ozone and of dietary α-tocopherol on the occurrence in rat lung of lipid peroxidation and alteration of the activity of enzymes important in detoxification of lipid peroxides were studied. Exposure to 0.7 and 0.8 ppm ozone continuously for 5 and 7 days, respectively, significantly elevated the concentration of TBA reactants, primarily malonaldehyde, produced by lipid peroxidation, as well as the activities of glutathione (GSH) peroxidase, GSH reductase and glucose-6-phosphate (G-6-P) dehydrogenase. As a logarithmic function of dietary α-tocopherol (0, 10.5, 45, 150 and 1500 mg/kg), the increase in formation of malonaldehyde and the increase in activities of GSH peroxidase and G-6-P dehydrogenase were partially inhibited. The activity of GSH reductase was not affected by dietary α-tocopherol. The concentration of malonaldehyde and the activity of GSH peroxidase in lung were linearly correlated ($p < 0.001$). This study confirmed the occurrence of lipid peroxidation in the lung during ozone exposure and revealed an enzymatic mechanism against damage. An apparent compensation mechanism is that with increased lipid peroxides there is increased activity of GSH peroxidase, which in turn increases lipid peroxide catabolism. The increased activities of GSH reductase and G-6-P dehydrogenase also function in the protective chain by providing increased levels of GSH and NADPH, respectively.

## INTRODUCTION

Malonaldehyde is derived from lipid peroxides and has been detected in tissue extracts (1-4). However the failure to find increasing amounts of lipid peroxides in the tissue of vitamin E-deficient or other nutritionally stressed animals has initiated a controversy as to whether lipid peroxides are formed in vivo (5). Nevertheless present knowledge favors the conclusion that small amounts of lipid peroxides are formed in vivo.

In a study of rat liver peroxidase, Little and O'Brien (6) demonstrated an intracellular glutathione (GSH) peroxidase (glutathione-hydrogen peroxide oxidoreductase, E.C. 1.11.1.9) that utilized lipid peroxide substrates. Christophersen (7,8) reported that the oxidation of glutathione by linoleate and linolenate hydroperoxides is enzymatically catalyzed in rat liver, and the products formed from the lipid peroxides are their corresponding monohydroxy polyenoic fatty acids. Both reports suggest that the enzymic reaction is probably responsible for the decomposition of most of the lipid peroxides in the liver cell and thus protects the cellular components from the deleterious effects of lipid peroxides.

Lipid peroxides are highly toxic and damaging to biological systems (9-13); however intravenously injected lipid peroxides are rapidly metabolized (14). Animals develop a tolerance to oxidants following prior exposure to sublethal dosages (15-17), and α-tocopherol and other antioxidants show limited effectiveness in the inhibition of lipid peroxidation damage in vivo (18-20). These facts indicate that animal tissue may have other mechanisms for protection against the toxic effect of lipid peroxides through their increased catabolism.

The damaging effects of nitrogen dioxide and ozone are related to their oxidative reactions, including initiation of lipid peroxidation, which can be partially prevented by α-tocopherol and other antioxidants (18-20). To understand how animals respond biochemically under ozone stress, these studies investigated alterations of lung tissue enzyme activities that may be important in the detoxification of lipid peroxides. An enzymatic protective mechanism against lipid peroxidation damage was found as a result of these studies.

## EXPERIMENTAL PROCEDURES

### Experiment I

Five groups of 1-month-old male Sprague-Dawley rats were fed a basal tocopherol-deficient diet (21) containing 15% stripped

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corn oil and 0, 10.5, 45, 150 and 1500 mg dl-α-tocopherol acetate per kilogram of diet, respectively. After 30 days, nine animals from the first dietary group and six from each of the other groups were exposed to 0.70 ± 0.15 ppm ozone continuously for 5 days, except for ca. 15 min daily during which time the chambers were cleaned and the diets replaced. Ozone was admitted to the chambers by a controlled flow device with 10 volume changes per hour. Ozone was monitored continuously by an ozone meter and at intervals by the neutral buffered KI method (22). This research is part of a program study at the University of California, Davis, on pulmonary effects of environmental oxidants. The ozone chambers are operated for this program research. Four rats from each dietary group served as controls. Control animals were placed in ambient air chambers where the highest 1 hr ambient oxidant level was 0.12 ppm. The animals of exposed and control groups were then sacrificed by withdrawal of blood via heart puncture following anesthetization by peritoneal injection of Beuthansia Special (Burns Pharmaceuticals). The lungs were removed, rinsed with 0.15 M KCl, blotted and finely minced within 2 hr after exposure. About 250-300 mg of the minced tissue from each animal was homogenized in a Potter-Elvehjem homogenizer with 6 ml of isotonic potassium phosphate buffer, pH 7.0, and 0.1 ml of 0.6% α-tocopherol in ethanol. The homogenate was filtered by suction through two layers of cheesecloth, and the filtrate was immediately assayed by the thiobarbituric acid (TBA) test. Protein was determined by the method of Miller (23).

The remaining lung minces from two to three animals of the same dietary group were pooled, and a 10% homogenate was made in 0.25 M sucrose containing 1 mM EDTA. After centrifugation for 10 min at 750 x g, the supernatant was centrifuged at 105,000 x g for 60 min in a Beckman Model L ultracentrifuge. The soluble fraction was used for measurement of GSH peroxidase, GSH reductase (reduced-NAD(P):oxidized glutathione oxidoreductase, E.C. 1.6.4.2) (24), glucose-6-phosphate (G-6-P) dehydrogenase (D-glucose-6-phosphate:NADP oxidoreductase, E.C. 1.1.1.49) (25) and protein.

#### Thiobarbituric Acid Test

One and one-half milliliters of the filtrate and 1.5 ml of 0.8% 2-thiobarbituric acid (Sigma) in 10% trichloroacetic acid were thoroughly mixed in test tubes and then placed in boiling water for 10 min and allowed to cool. After centrifugation, the absorbance of the

TABLE I

Effects of $O_3$ Exposure and Dietary α-Tocopherol on Malonaldehyde Formation and Activities of GSH Peroxidase, GSH Reductase and G-6-P Dehydrogenase (Experiment 1)[a]

| Dietary α-tocopherol, mg/kg diet | TBA reactants, nmoles malonaldehyde/g protein | | | GSH peroxidase, nmoles GSH oxidized/min/mg protein | | | GSH reductase, nmoles NADPH oxidized/min/mg protein | | | G-6-P dehydrogenase, nmoles NADPH formed/min/mg protein | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $O_3$ Exposed | Control | $p$ | $O_3$ Exposed | Control | $p$ | $O_3$ Exposed | Control | $p$ | $O_3$ Exposed | Control | $p$ |
| 0 | 162 ± 19[b] | 82 ± 18 | <0.001 | 50 ± 4 | 30 ± 0 | <0.01 | 118 ± 9 | 87 ± 7 | <0.05 | 236 ± 7 | 154 ± 11 | <0.01 |
| 10.5 | 134 ± 15 | 70 ± 9 | <0.001 | 39 ± 5 | 26 ± 3 | <0.05 | 114 ± 9 | 107 ± 13 | >0.1 | 216 ± 29 | 158 ± 7 | <0.1 |
| 45 | 125 ± 10 | 54 ± 14 | <0.001 | 39 ± 4 | 24 ± 5 | <0.05 | 116 ± 9 | 98 ± 6 | <0.1 | 210 ± 17 | 156 ± 9 | <0.05 |
| 150 | 122 ± 22 | 54 ± 18 | <0.001 | 37 ± 1 | 24 ± 3 | <0.01 | 125 ± 8 | 105 ± 15 | <0.1 | 209 ± 17 | 159 ± 9 | <0.05 |
| 1500 | 106 ± 11 | 40 ± 0 | <0.001 | 27 ± 8 | 20 ± 4 | <0.1 | 118 ± 4 | 107 ± 15 | >0.1 | 103 ± 12 | 165 ± 2 | <0.1 |
| $\bar{X}$[c] | 113 ± 25 | 63 ± 19 | <0.001 | 38 ± 8 | 25 ± 4 | <0.001 | 118 ± 8 | 101 ± 11 | <0.001 | 213 ± 21 | 159 ± 7 | <0.01 |

[a]GSH = Glutathione; G-6-P = glucose-6-phosphate.

[b]*Mean ± standard deviation.*

[c]Mean ± standard deviation for all dietary groups.

TABLE II

Effects of $O_3$ Exposure and Dietary α-Tocopherol on Malonaldehyde Formation and Activities of GSH Peroxidase, GSH Reductase and G-6-P Dehydrogenase (Experiment II)[a]

| Dietary α-tocopherol, mg/kg diet | TBA reactants, nmoles malonaldehyde/g protein | | | GSH peroxidase, nmoles GSH oxidized/min/mg protein | | | GSH reductase, nmoles NADPH oxidized/min/mg protein | | | G-6-P dehydrogenase, nmoles NADPH formed/min/mg protein | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $O_3$ Exposed | Control | $p$ | $O_3$ Exposed | Control | $p$ | $O_3$ Exposed | Control | $p$ | $O_3$ Exposed | Control | $p$ |
| 0 | 105 ±15[b] | 80 ± 7 | <0.001 | 32 ±4 | 19 ±2 | <0.001 | 94 ±10 | 81 ±7 | <0.001 | 173 ±27 | 121 ±11 | <0.001 |
| 45 | 90 ±13 | 78 ± 10 | <0.01 | 28 ±4 | 19 ±2 | <0.001 | 96 ± 6 | 83 ±7 | <0.001 | 147 ±17 | 121 ±10 | <0.001 |
| $p$[c] | <0.02 | >0.1 | | <0.01 | >0.1 | | >0.1 | >0.1 | | <0.01 | >0.1 | |
| $\bar{X}$[d] | 98 ±16 | 79 ± 9 | <0.001 | 30 ±4 | 19 ±2 | <0.001 | 95 ± 8 | 82 ±7 | <0.001 | 160 ±26 | 121 ±10 | <0.001 |

[a]See Table I.
[b]Mean ± standard deviation.
[c]Probability value of 0 vs. 45 mg α-tocopherol/kg diet.
[d]Mean ± standard deviation of all dietary groups.

clear supernatant was measured at 532 nm, and the amount of TBA reactants was calculated based on the molar extinction coefficient of malonaldehyde ($1.56 \times 10^5$).

### Preparation of Ethyl Linolenate Hydroperoxide

The hydroperoxide was prepared by oxidation of ethyl linolenate in hexane at room temperature according to a modification of the procedure of Banks et al. (26). Crude ethyl linolenate hydroperoxide, obtained by stripping the solution with 87% ethanol, was purified by thin layer chromatography on Silica Gel G using petroleum ether-ether-acetic acid 8:7:0.5 v/v, or 1% methanol in benzene, or both, as solvent system. The peroxide concentration was determined spectrophotometrically in ethanol at 232 nm, and calculations were made using the molar extinction coefficient, $2.5 \times 10^4$ (27).

### Glutathione Peroxidase Assay

The peroxidase activity was assayed at 25C with glutathione as hydrogen donor using a system containing 0.1 M Tris-HCl buffer, pH 7.6, 200 μM EDTA, 650 μM GSH, 350 μM ethyl linolenate hydroperoxide and ca. 1 mg of enzyme protein in a final volume of 1.0 ml. The reaction was started by addition of the peroxide and was stopped by denaturing the enzyme with 4 ml of 6.3% trichloroacetic acid. The reduced glutathione remaining was measured according to a modification of the procedure of Sedlack and Lindsay (28). The colorimetric determination was carried out by mixing 2 ml of the clear supernatant with 2 ml of 0.4 M Tris-HCl buffer, pH 8.9 and 0.1 ml of 10 mM, 5,5-dithiobis-(2-nitrobenzoic acid) in methanol. The absorbance was read within 5 min after the reaction. Control samples without addition of the peroxide or enzyme, or both, were also determined. A molar extinction coefficient of $1.36 \times 10^4$ for GSH (29) was used for calculations.

### Experiment II

Thirty and twenty-nine 1-month-old male Sprague-Dawley rats (chronic respiratory disease-free) were maintained on a basal diet containing 0 and 45 mg dl-α-tocopherol acetate per kilogram, respectively. These diets were the same as diets 1 and 3 of Experiment I. After 35 days, 15 animals from each group were exposed to 0.79 ± 0.14 ppm ozone continuously for 7 days. The exposed and control animals were then sacrificed. The lung samples were prepared and assayed as described in Experiment I, except samples were not pooled.

## RESULTS

### Occurrence of Lipid Peroxidation Following Ozone Exposure

Tables I and II show the results of Experiments I and II, respectively. The concentration of malonaldehyde, as measured by TBA reactants, in the filtered lung homogenates of all dietary groups was significantly increased by whole animal exposure to ozone. The increases averaged 80% and 24% for Experiments I and II, respectively. The increase of malonaldehyde was partially inhibited by dietary α-tocopherol. Figure 1A shows that in Experiment I the concentration of malonaldehyde decreased linearly as a function of the logarithm of dietary α-tocopherol, with $r$ values of ca. -0.8. Relatively little effect of α-tocopherol in the control group of Experiment II was observed.

The sensitivity of the measurement of malonaldehyde was enhanced by using a large sample volume (up to 100 mg of homogenized tissue in a 3 ml reaction volume). The formation of artifacts due to in vitro lipid peroxidation was minimized by analyzing the α-tocopherol-protected homogenates immediately after their preparation.

### Effect of Ozone and α-Tocopherol on the Activity of GSH Peroxidase

Tables I and II show that the activity of GSH peroxidase in the soluble fraction of lung was significantly elevated by whole animal exposure to ozone in all dietary groups. These increases averaged 52% and 58% for Experiments I and II, respectively. Alteration of the enzymic activity was also modified by dietary α-tocopherol. Figure 1B shows the relationship of GSH peroxidase activity to α-tocopherol concentration in Experiment I. The data indicate that as a logarithmic function of concentration α-tocopherol partially inhibited the elevation of the enzymic activity due to ozone treatment. For the ozone exposed groups $r$ = -0.888 and $p < 0.001$; for the control groups $r$ = -0.809 and $p < 0.01$.

### Effect of Ozone and α-Tocopherol on the Activity of GSH Reductase

The activity of GSH reductase in the soluble fraction of lung (Tables I and II) was also significantly elevated in all dietary groups following ozone exposure. These increases averaged 16% and 17% for Experiments I and II, respectively. The activity of the enzyme was not altered by dietary α-tocopherol.

### Effect of Ozone and α-Tocopherol on the Activity of G-6-P Dehydrogenase

Tables I and II also show that exposure of animals to ozone markedly increased the activity of lung G-6-P dehydrogenase in all dietary groups. The increases averaged 33% and 32% for Experiment I and II, respectively. In Experiment I, dietary α-tocopherol partially, but linearly ($r$ = -0.681, $p$ = <0.01), retarded the alteration of the enzymic activity due to ozone exposure. There was no effect of α-tocopherol on G-6-P dehydrogenase activity in the lung of control animals. Similar results were observed in Experiment II.

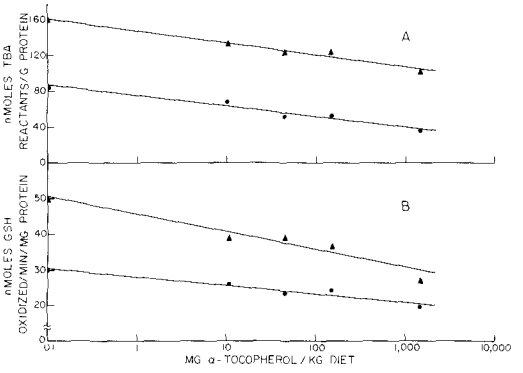


FIG. 1. Linear relationships of logarithm of α-tocopherol concentration with (A) malonaldehyde concentration (ozone exposed animals $r$ = -0.787 and $p < 0.001$; control animals $r$ = -0.773 and $p < 0.001$) and (B) glutathione peroxidase activity (ozone exposed animals $r$ = -0.888 and $p < 0.001$; control animals $r$ = -0.809 and $p < 0.001$). (▲), ozone exposed; (●), control.

### Relationship of Malonaldehyde Formation with GSH Peroxidase Activity

Figure 2 (Experiment I) and Figure 3 (Experiment II) are plots of concentration of malonaldehyde vs. the activity of GSH peroxidase in lung tissue. The linear regression coefficient of the malonaldehyde content and the activity of GSH peroxidase ($r$ = 0.848 and 0.661 for Experiments I and II, respectively) of lung indicate that these two measurements are highly correlated ($p < 0.001$ for both experiments).

## DISCUSSION

Whole animal exposure to ozone elevated the concentration of the lipid peroxidation product malonaldehyde as well as GSH peroxidase activity in the lung tissue, and dietary α-tocopherol partially reversed the elevation of each. The finding that α-tocopherol is partially effective in the inhibition of the formation of lipid peroxidation products confirms that it functions as a biological antioxidant and that lipid peroxidation occurred in vivo during

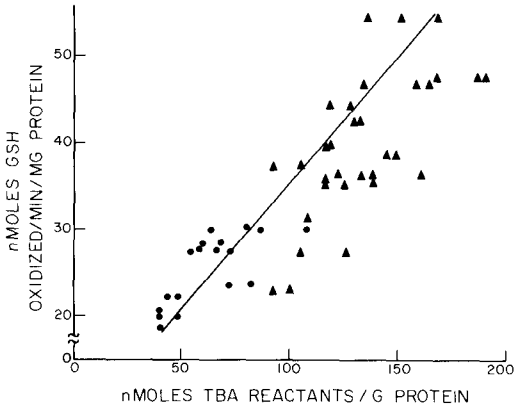


FIG. 2. Correlation between malonaldehyde concentration and glutathione peroxidase activity for Experiment I in all ozone exposed (▲) and all control (●) animals; $r = 0.848$, $p < 0.001$.

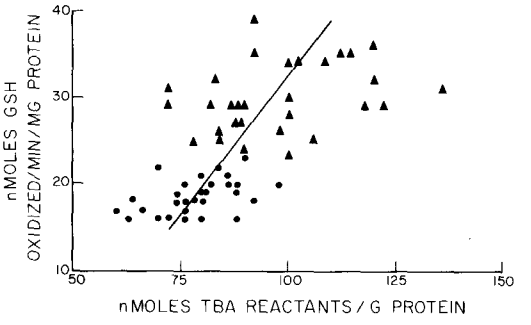


FIG. 3. Correlation between malonaldehyde concentration and glutathione peroxidase activity for Experiment II in all ozone exposed (▲) and all control (●) animals; $r = 0.661$, $p < 0.001$.

ozone exposure. The high correlation between the concentration of malonaldehyde and the activity of GSH peroxidase suggests that the enzyme activity is probably affected by lipid peroxide formation. Whether the increase of GSH peroxidase activity is due to an altered rate of synthesis or to other changes remains to be shown.

The observed elevated activity of GSH reductase following exposure of animals to ozone may be attributable to the increased demand for reduced glutathione as a result of the increased activity of GSH peroxidase. Since dietary α-tocopherol did not modify the GSH reductase activity and since there was a lack of correlation between the activity of this enzyme and that of GSH peroxidase, direct oxidation of GSH by the oxidant may also have contributed to alteration of the enzyme activity. α-Tocopherol does not protect sulfhydryl compounds from direct oxidation.

The increase of G-6-P dehydrogenase activity during ozone exposure reflects the increased oxidation of NADPH due to elevated activity of GSH reductase and perhaps in part the direct oxidation of NADPH. The reported antioxigenic action of GSH and other sulfhydryl compounds (30,31), therefore, could be attributable to their ability to become proton donors for GSH peroxidase. Dietary α-tocopherol partially inhibited the increase of the G-6-P dehydrogenase activity, suggesting that the vitamin might have some relation to this system.

Pinto and Bartley (32) observed that GSH peroxidase activity in liver of male rats increased steadily from foetal stage up to ca. 55 days and then remained unchanged, while GSH reductase activity went up and down during the weaning period and reached a plateau at ca. 45 days. Increased G-6-P dehydrogenase activity has been observed in rat red blood cells during hyperoxia (33) and in rat liver upon starvation and refeeding with a carbohydrate diet containing adequate protein (34,35).

The results reveal that rat lung has an enzymic pathway for reducing lipid peroxides (Fig. 4) in vivo. The ability of animals to respond to an increase of lipid peroxide by increasing the activity of GSH peroxidase, which results in increased peroxide catabolism, is the key feature of the mechanism. Harmless hydroxy fatty acids formed by reduction of lipid peroxides (7,8) can be metabolized via the β-oxidation pathway. The increases in the activity of GSH reductase and G-6-P dehydrogenase are also parts of the protective chain by providing the increased requirement for GSH and NADPH, respectively.

Beutler (36) summarized a mechanism in human erythrocytes for detoxification of hydrogen peroxide formed by oxidative drug metabolism involving GSH peroxidase, GSH reductase and G-6-P dehydrogenase in the maintenance of reduced GSH and the decomposition of $H_2O_2$. When the cells genetically lack one of these enzymes (37-39) or enzymes involved in the maintenance of GSH levels, such as GSH synthetase (40,41), they become susceptible to drug-induced hemolytic anemia. The lack of specificity of rat liver GSH peroxidase using a variety of hydroperoxides as substrate (6) suggests that this is probably a type of protective mechanism similar to that observed in the lung. Genetic deficiency of one of these enzymes would be expected to cause increased susceptibility to lipid peroxidation damage. The findings suggest that the protective mechanism against lipid peroxidation damage is not confined to the lung tissue or to any particular species. Therefore the presence of the suggested

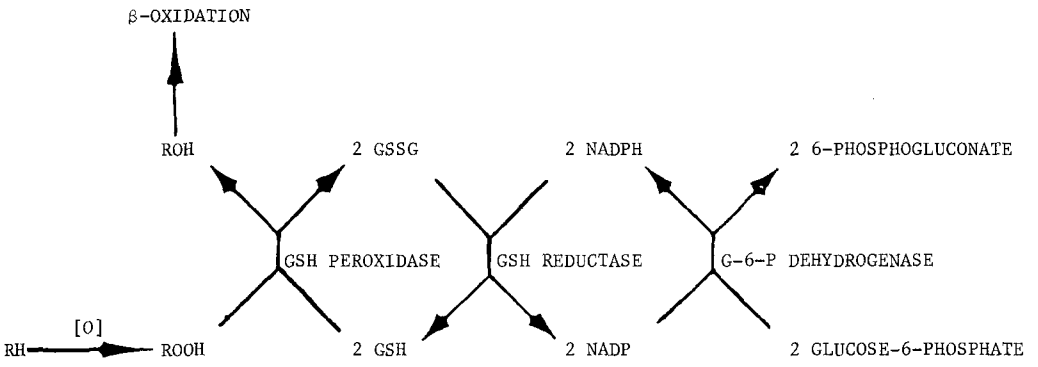


FIG. 4. Scheme of lipid peroxide metabolism. RH, polyunsaturated fatty acid; ROOH, fatty acid hydroperoxide; ROH, hydroxy fatty acid; GSH, reduced glutathione; GSSG, oxidized glutathione.

enzymatic protective mechanism against lipid peroxidation damage may explain the following observations: (a) failure to detect any or to detect increasing amounts of lipid peroxide in nutritionally stressed animals (5); (b) development of tolerance by animals to oxidants, e.g., ozone and $NO_2$, following prior sublethal exposure (15-17); (c) insignificant decrease of SH groups following ozone exposure (Chow and Tappel, unpublished); and (d) rapid metabolism of injected lipid peroxide by the animal (14). The same type of protective mechanism may also apply to lipid peroxidation induced by $NO_2$ exposure (19,20) and hyperoxia (42), as well as $CCl_4$ (43), ethanol (44) and other free radical inducing agents. While the glutathione peroxidase pathway appears to be a major protective mechanism, other types of protective mechanisms would work in parallel.

Alteration in tissue enzyme activity is a more sensitive and specific indicator of tissue damage than other known biochemical measurements. Hence the alteration of the activity of protective enzymes, GSH peroxidase in particular, should be useful as an index for monitoring oxidant damage to lung and the occurrence of lipid peroxidation in vivo.

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