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DECREASE IN MOUSE LUNG AND LIVER GLUTATHIONE PEROXIDASE ACTIVITY AND POTENTIATION OF THE LETHAL EFFECTS OF OZONE AND PARAQUAT BY THE SUPEROXIDE DISMUTASE INHIBITOR DIETHYLDITHIOCARBAMATE

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Abstract—Intraperitoneal injection of mice with sodium diethyldithiocarbamate (1.2 g/kg), an inhibitor of superoxide dismutase, potentiated the lethal effects of ozone and of paraquat. Study of control mice injected with sodium diethyldithiocarbamate demonstrated an 80 per cent decrease in lung and liver superoxide dismutase activity in one hour, followed by a slow return towards normal. However, liver and lung glutathione peroxidase activites were also decreased, although to a lesser extent than superoxide dismutase, and the nadir was not observed until six hours and 30 min following injection. A decrease in liver non-protein sulfhydryl groups was also observed. Similarly, incubation of liver homogenate with diethyldithiocarbamate produced a marked loss in superoxide dismutase activity associated with a lesser, delayed decrease in gluthione peroxidase. The latter could be prevented by the addition of superoxide dismutase or by anaerobic conditions. Accordingly, the potentiation of the lethal effects of exogenous agents by diethyldithiocarbamate does not necessarily indicate a role for superoxide anion radical in the toxicity of these agents.

Superoxide anion radical (O_2^-) is believed to be produced in a number of normal endogenous processes including the autoxidation of hemoglobin, microsomal mixed-function oxidase hydroxylations, and in association with granulocyte phagocytosis [1–3]. This highly reactive radical has also been suggested to be responsible for the toxicity of various exogenous agents including ionizing radiation [4], hemolytic drugs [5], pulmonary irritants such as paraquat [6], ozone [7] and hyperbaric oxygen [8], and the neurotoxin 6-hydroxy-dopamine [9]. However, the evidence favoring the role of superoxide anion radical in these processes tends to be indirect in that this active species can not be measured *in vivo*. One approach to evaluating the *in vivo* effects of O_2^- would be to specifically inhibit superoxide dismutase, thereby presumably intensifying the effect of any agent which acts through O_2^- formation. Heikkila *et al.* [10] have suggested that sodium diethyldithiocarbamate (DDC), which had previously been shown to inhibit purified superoxide dismutase [11, 12] might be a suitable probe for this purpose. These authors reported that brain, liver, and blood superoxide dismutase is inhibited in a dose dependent fashion by diethyldithiocarbamate following intraperitoneal injection in mice. They further demonstrated an almost total loss in activity of this enzyme following incubation of brain and liver homogenates or blood lysates with diethyldithiocarbamate. More recently, DDC has been found to potentiate oxygen toxicity and the *in vitro* effects of hemolytic agents producing O_2^- [13, 14].

In addition to superoxide dismutase, there are other enzymes and intermediates which are believed to protect against the effects of active oxygen species. Among these are catalase and the glutatione peroxidase system, including reduced glutathione. In the case of ozone toxicity, the importance of glutathione peroxidase is suggested by studies demonstrating a dose dependent elevation in the activity of this enzyme following ozone exposure, and evidence indicating that increased activity of this enzyme may be responsible for tolerance to subsequent ozone exposure [15, 16]. There is also evidence suggesting that sulfhydryl compounds play an important protective role against ozone toxicity, as well as the toxicity of other oxidizing agents [17, 18].

In the present study we have shown that injection of DDC produces inhibition of lung superoxide dismutase and potentiation of the lethal effects of ozone and of paraquat. However, interpretation of these results in relation to O_2^- is complicated by the observation that injection of DDC also results in a decrease in the activity of glutathione peroxidase, and in non-protein sulfhydryl levels.

METHODS

Female mice (CF-1: Carsworth Laboratories), 25–30 g, were used throughout. Mice were exposed in a Plexiglas chamber to ozone as previously described [19]. Ozone levels were measured by a chemiluminescent monitor. Three experiments were performed in which half of the animals were injected intraperitoneally with 1.2 g/kg of sodium diethyldithiocarbamate (DDC: Sigma Chemical Company) in

isotonic phosphate buffered (0.01 M) saline, pH 7.4, 30 min prior to ozone exposure while the remaining half received buffer alone. The time of death was recorded for each animal during continuous exposure to 3.6–4.2 ppm ozone. The data were analyzed by multiplying the time of death in hours by the ozone concentration to obtain ppm-hr of exposure for each mouse. In control experiments this level of DDC did not by itself produce death. For study of paraquat toxicity, 1.2 g/kg of DDC was injected intramuscularly 30 min before intraperitoneal injection of paraquat (gift of Dr. M. S. Rose, Imperial Chemical Industries, England). The mice were observed for seven days and the number of deaths recorded.

Studies of lung and liver superoxide dismutase, catalase, and glutathione peroxidase activities, and of non-protein sulfhydryl levels, were performed in control mice injected intraperitoneally with 1.2 g kg diethyldithiocarbamate. At specified times following injection, the mice were decaptitated and the liver and both lungs were removed. After a gentle rinse in cold saline, the organs were blotted dry, weighed, and homogenized in 10 vol. of 0.05 M potassium phosphate buffer, pH 6.5 with 10^{-4} M EDTA. The homogenate was centrifuged at 900 *g* for 10 min at 4 and the supernatant removed and stored in an ice bucket prior to assay. Where indicated, the soluble supernatant fraction of whole liver homogenate was obtained by centrifugation at 100,000 *g* for 1 hr.

Superoxide dismutase activity was measured in triplicate using 6-hydroxy-dopamine–HBr (Aldrich Chemical Co.) [10]. In some studies the results were confirmed with the epinephrine autoxidation assay of Misra and Fridovich [20]. Standard curves using purified superoxide dismutase (Truett Laboratories) were obtained during each set of assays. Glutathione peroxidase activity was assayed using cumene hydroperoxide as a substrate by coupling to NADPH via added glutathione reductase [21]. The rate of NADPH oxidation was measured spectrophotometrically at 340 nm and corrected for blanks containing either no tissue or no cumene hydroperoxide. Catalase activity was measured by a procedure in which the rate of decomposition of added H_2O_2 is determined by spectrophotometric assay of residual H_2O_2 through reaction with a standard excess of $KMnO_4$ [22]. Measurement of non-protein sulfhydryl groups was performed by reaction with 5,5′-dithiobis (2-nitrobenzoic acid) following deproteinization with trichloroacetic acid [23]. In studies of the *in vitro* effects of DDC the homogenates were incubated at 37° in a heating block. Studies under nitrogen were performed in a disposable glove bag inflated with prepurified nitrogen bubbled through alkaline pyrogallol to remove residual oxygen.

RESULTS

The mean ± S.E. survival of 20 mice injected with DDC was 21.3 ± 0.7 ppm-hr as compared to 26.4 ± 1.2 ppm-hr in the 20 buffer-injected control mice (<0.01) simultaneously inhaling the same ozone concentrations. Log probit analysis of the data revealed no statistically significant difference in the slopes for both groups. Injection of DDC also potentiated the lethal effects of paraquat. Deaths were observed in three of twenty mice receiving the reported LD_{25} dose of 25 mg/kg paraquat [24], and in eighteen of twenty mice injected with both this level of paraquat and 1.2 mg/kg DDC ($P < 0.01$). There were no deaths in the twenty mice injected with diethyldithiocarbamate alone.

Lung and liver superoxide dismutase activity in mice injected with DDC was decreased by close to 80 per cent in 1 hr with a slow return towards normal by the next day (Table 1). This is similar to the observations of Heikkila *et al.* in brain, liver, and red cells [10]. DDC also produced a decrease in glutathione peroxidase activity which occurred more rapidly and to a greater extent in liver than in lung (Table 1). In both organs the decrease in glutathione peroxidase activity was not as pronounced as the initial loss in superoxide dismutase activity, and the nadir did not occur until 6.5 hr after injection.

Liver non-protein sulfhydryl levels were 5.87 ± 0.37 μmol/g protein in the control animals and 3.82 ± 0.37 and 4.35 ± 0.33 μmol/g protein at 4 and 6.5 hr, respectively, following injection of DDC ($P < 0.05$). Lung non-protein sulfhydryl levels were also affected but to a lesser extent (control level 2.27 ± 0.16; 4 hr post DDC 1.98 ± 0.19; 6.5 hr post DDC 1.85 ± 0.20 μmol/g protein). No consistent or significant effect was noted on lung or liver catalase activity which ranged from 82–108 per cent of control at different time periods.

Incubation of liver and lung homogenates (2 mg protein/ml) with 0.01 M DDC produced decreases in enzyme activity similar to that observed following

Table 1. Effect of intraperitoneal injection of diethyldithiocarbamate (1.2 g/kg) on mouse liver and lung superoxide dismutase and glutathione peroxidase. Each number represents the mean ± S.E. of 6–12 mice calculated as percent of control value*

Time (hr)	Liver		Lung	
	Superoxide dismutase	Glutathione peroxidase	Superoxide dismutase	Glutathione peroxidase
0	100 ± 4.2	100 ± 7.0	100 ± 6.6	100 ± 11.5
1	17.6 ± 2.7	66.5 ± 4.6	20.4 ± 2.3	96.9 ± 12.3
4	16.5 ± 1.9	39.6 ± 4.8	23.1 ± 2.7	71.5 ± 7.3
6.5	28.1 ± 1.5	29.6 ± 6.0	24.6 ± 3.8	56.9 ± 10.4
22	57.7 ± 1.9	96.5 ± 11.5	63.8 ± 3.8	106.5 ± 7.3

*Control glutathione peroxidase activity in liver was 316 ± 22 and in lung was 78 ± 9 nmol NADPH oxidized/min/mg protein. Control superoxide dismutase levels were 639 ± 27 μg/g in liver and 168 ± 11 μ/g in lung.

Table 2. Glutathione peroxidase activity in liver fractions incubated at 37° for 6.5 hr in air or in nitrogen with or without sodium diethyldithiocarbamate (DDC: 10 mM), superoxide dismutase (SOD: 200 μg/ml) or dihydroxyfurmaric acid (DHF: 11.2 mM)

Incubation conditions	Enzyme activity* nmoles NADPH oxidized/min/mg protein	% of control
900 *g* supernatant		
air	328 ± 26	100 ± 8
air: DDC	106 ± 15	32 ± 14
air: DDC: SOD†	302 ± 38	92 ± 13
nitrogen	318 ± 63	97 ± 20
nitrogen: DDC	288 ± 48	88 ± 17
100,000 *g* supernatant		
air	350 ± 18	100 ± 5
air: DDC	273 ± 37	78 ± 14
air: DDC; DHF	184 ± 13	53 ± 7

*Expressed as mean ± S.E.
†SOD added 120 min after start of incubation.

in vivo administration. Less than 20 per cent of initial superoxide dismutase activity was observed in both liver and lung after 1.5, 4 and 6.5 hr of incubation as compared to parallel controls. Lung and liver glutathione peroxidase activity was unaffected at 1.5 hr of incubation (98 and 106 per cent of control, respectively). Liver glutathione peroxidase had decreased to 44 per cent of control activity after 4 hr of incubation and 32 per cent after 6.5 hr. As the *in vivo* studies, there was less of an effect on lung glutathione peroxidase which was 92 per cent of control activity after 4 hr and 72 per cent after 6.5 hr of incubation.

Performance of the incubation procedure under nitrogen, which produced a similar decrease in superoxide dismutase activity, protected against the loss in glutathione peroxidase activity in liver homogenates containing DDC. Protection was also afforded by the addition of superoxide dismutase two hours after beginning the incubation (Table 2). A lesser decrease in glutathione peroxidase activity was noted when the 100,000 *g* supernatant of the liver homogenate was incubated with DDC although there was again a greater than 80 per cent decrease in superoxide dismutase activity (Table 2). Addition of dihydroxyfumaric acid, an agent reported to produce O_2^- [25], resulted in a more pronounced loss of glutathione peroxidase activity.

DISCUSSION

Injection of mice with the superoxide dismutase inhibitor DDC potentiates the lethal effects of both paraquat and ozone. Superoxide anion radical formation has been previously demonstrated in lung homogenates incubated with paraquat [6]. The evidence suggesting a role for O_2^- in the toxicity of ozone is more indirect, being based on the observation that rats exposed to 0.8 ppm ozone for 7 days have an increase in lung superoxide dismutase activity [7]. However, this might be a nonspecific response reflecting the replacement of type I by more metabolically active type II alveolar cells which occurs at this level of ozone exposure [26]. Furthermore, while ozone has been suggested to be a product of the gaseous decomposition of superoxide anion radical, a chemical mechanism for the formation of O_2^- from ozone is not apparent.

The present study demonstrates that the superoxide dismutase inhibitor DDC produces a decrease in the activity of glutathione peroxidase both *in vitro* and *in vivo*. The mechanism of this effect may be related to the formation of endogenous O_2^-. This is suggested by the finding that the decrease in lung and liver glutathione peroxidase occurs later than that of superoxide dismutase activity, by the prevention of loss in glutathione peroxidase activity provided by both anaerobic conditions and added superoxide dismutase, and by the potentiation of this effect produced by dihydroxyfurmarate. Microsomal production of O_2^- might possibly account for the greater inhibition of glutathione peroxidase in the 900 *g* than in the 100,000 *g* liver supernatant as well as the more pronounced effect in liver than in lung. However, alternate explanations for the DDC-induced decrease in glutathione peroxidase have not been precluded.

The loss in glutathione peroxidase activity, as well as the decrease in non-protein sulfhydryl groups, greatly complicates interpreting the potentiation by DDC of lethal effects due to ozone and paraquat in relation to a possible role for O_2^- in the toxicity caused by these agents. A decrease in glutathione peroxidase activity or in non-protein sulfhydryl groups would presumably exacerbate the effects of oxidizing agents independent of the presence of O_2^-. Accordingly, the use of DDC as a probe for the *in vivo* role of O_2^- produced by exogenous factors would appear to be limited.

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REFERENCES

1. I. Fridovich, *A. Rev. Biochem.* **55**, 147 (1975).
2. R. E. Lynch, R. G. Lee and G. E. Cartwright, *J. biol. Chem.* **251**, 1015 (1976).
3. B. M. Babior, R. S. Kipnes and J. T. Curnutte. *J. clin. Invest.* **52**, 741 (1973).
4. A. Petkau, K. Kelly, W. S. Chelack, S. D. Pleskach, C. Barefoot and B. E. Meeker, *Biochem. biophys. Res. Commun.* **67**, 1167 (1975).
5. B. Goldberg, A. Stern and J. Peisach, *J. biol. Chem.* **251**, 3045 (1976).
6. J. S. Bus, S. D. Aust and J. E. Gibson, *Biochem biophys. Res. Commun.* **58**, 749 (1974).
7. M. G. Mustafa, *Clin. Res.* **23**, 138A (1975).
8. J. D. Crapo and D. F. Tierney, *Am. J. physiol.* **225**, 1401 (1974).
9. G. Cohen and R. E. Heikkila, *J. biol. Chem.* **249**, 2447 (1974).
10. R. E. Heikkila, F. S. Cabbat and G. Cohen, *J. biol. Chem.* **251**, 2182 (1976).
11. K. Asada, M. Takahashi and M. Nagate, *Agric. biol. Chem.* **38**, 471 (1974).
12. H. P. Misra *Fedn Proc.* **34**, 624A (1975).
13. L. Frank, D. L. Wood and R. J. Roberts, *Biochem. Pharmac.* **27**, 251 (1978).
14. B. Goldberg and A. Stern, *J. biol. Chem.* **251**, 6468 (1976).
15. C. K. Chow and A. L. Tappel, *Lipids* **7**, 518 (1972).
16. C. K. Chow *Nature, Lond.* **260**, 721 (1976).
17. A. J. DeLucia, P. M. Hoque, M. G. Mustafa and C. E. Cross, *J. Lab. clin. Med.* **80**, 559 (1972).
18. E. J. Fairchild, S. D. Murphy and H. E. Stokinger, *Science, N.Y.* **130**, 861 (1959).
19. B. D. Goldstein and O. J. Balchum, *Toxic. appl. Pharmac.* **27**, 330 (1974).
20. H. P. Misra and I. Fridovich, *J. biol. Chem.* **247**, 3170 (1972).
21. C. Little, R. Olinescu, K. G. Reid and P. J. O'Brien, *J. biol. Chem.* **245**, 3632 (1970).
22. G. Cohen, D. Dembiec and J. Marcus, *Analyt. Biochem.* **34**, 30 (1970).
23. J. Sedlak and R. H. Lindsay, *Analyt. Biochem.* **25**, 192 (1968).
24. J. S. Bus, S. Z. Cagen, M. Olgaard and J. E. Gibson, *Toxic. appl. Pharmac.* **35**, 501 (1976).
25. B. Goldberg and A. Stern, *Archs. Biochem. Biophys.* **178**, 218 (1977).
26. R. J. Stephens, M. F. Sloan, M. J. Evans and G. Freeman, *Am. J. Path.* **74**, 31 (1974).