0022-3565/80/2123-0452$02.00/0
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS
Vol. 212, No. 3

Mechanism for the Potentiation of Oxygen Toxicity by Disulfiram[1]

HENRY JAY FORMAN, JONATHAN L. YORK and ARON B. FISHER[2]

Department of Physiology, School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania

Accepted for publication December 4, 1979

ABSTRACT

Forman, Henry Jay, Jonathan L. York and Aron B. Fisher: *Mechanism for the potentiation of oxygen toxicity by disulfiram.* J. Pharmacol. Exp. Ther. **212:** 452–455, 1980.

Rats given disulfiram (200 mg/kg) or diethyldithiocarbamate (200 mg/kg) by intraperitoneal injection were exposed to 2 atmospheres absolute oxygen in a hyperbaric chamber or kept in normoxia. By 12 hr of hyperoxic exposure, none of the control but 30% of the disulfiram-treated and 87% of the diethyldithiocarbamate-treated rats had died. Both disulfiram and diethyldithiocarbamate administration decreased lung cytosolic superoxide dismutase activity, but the pharmacokinetics were different. At 1 hr postinjection of diethyldithiocarbamate superoxide dismutase activity was 40% decreased but returned to control activity within 13 hr (4 hr, 18% inhibited). In contrast, disulfiram administration produced a greater decrease at 4 hr (31%) than at 1 hr (16%) and was still effective at 13 hr (28% less than control). Although disulfiram did not produce as great a decrease at 1 hr as did diethyldithiocarbamate, it's effect was more persistent.

In vitro, diethyldithiocarbamate inactivated superoxide dismutase at 10^{-4} M, although 10^{-3} M disulfiram did not cause any reduction in enzymatic activity. The contrast between the inhibition by disulfiram of lung superoxide dismutase activity *in vivo* and its lack of effect *in vitro* suggests metabolism of disulfiram to diethyldithiocarbamate. It is likely that disulfiram administration potentiates oxygen toxicity *via in vivo* reduction to diethyldithiocarbamate and subsequent inhibition of superoxide dismutase.

Pulmonary damage is the cause of death when animals are exposed to O_2 pressures between 0.5 and 2.5 atmospheres absolute O_2, although animals breathing O_2 at higher pressures generally die with seizures and other manifestations of central nervous system toxicity (Clark and Lambertsen, 1971). Disulfiram [Antabuse, tetraethylthioperoxydicarboxic diamide, tetraethylthiuram disulfide, bis(diethylthiocarbamyl)disulfide], an agent introduced into clinical practice in 1948 by Hald *et al.* (1948) for treatment of chronic alcoholism has been shown to prevent seizures in animals due to hyperbaric oxygen exposure (Faiman *et al.*, 1971, 1974). This has led to a suggestion that disulfiram could be an effective drug to prevent toxicity associated with oxygen therapy. On the other hand, Deneke *et al.* (1979) have shown that disulfiram did not protect rats and actually enhanced pulmonary oxygen toxicity at 1 atmosphere absolute O_2. The mechanism of how disulfiram potentiates O_2 toxicity below the seizure threshold is the subject of this investigation.

Disulfiram, a disulfide, is converted *in vivo* into two molecules of diethyldithiocarbamate (DDC), its corresponding thiol. The latter compound is a potent inhibitor of cytosolic superoxide dismutase (SOD) both *in vitro* and *in vivo* (Heikkila *et al.*, 1976; Frank *et al.*, 1978). Previous studies have indicated that inhibition of lung cytosolic SOD by DDC correlated with decreased survival of rats in 1 atmosphere absolute O_2. It would seem possible that the mode of disulfiram enhancement of oxygen toxicity below the seizure threshold could be through conversion of disulfiram to DDC. In this study, we compared the effects of DDC and disulfiram on mortality of rats exposed to 2 atmospheres absolute O_2, a condition which accelerates lethal pulmonary toxicity but is below the seizure threshold for this species (Fisher *et al.*, 1979). We also measured the relative effects of 200 mg/kg of DDC or disulfiram on pulmonary superoxide dismutase activity as a function of time after administration. The evidence supports the hypothesis that disulfiram enhances O_2 toxicity because of conversion to DDC and subsequent inhibition of SOD.

Materials and Methods

Disulfiram (50 mg/ml) was suspended in 0.9% NaCl- (w/v) 5% Pluronic-F68 (w/v) and the pH was adjusted to 7.4 to 8.0. Solutions of DDC (50 mg/ml) were prepared similarly. Sprague-Dawley rats (205–390 g) (Charles River, Wilmington, MA) were administered either disulfiram (200 mg/kg b.wt.) or DDC (200 mg/kg b.wt.) by i.p. injection. Vehicle control animals were given an equivalent dose of NaCl-Pluronic-F68 solution. The rats were provided with a standard laboratory diet and water. Four groups of rats were injected with 200 mg/kg of disulfiram, or 200 mg/kg of DDC or the vehicle solution 1 hr before exposure to O_2 at 2 atmospheres or air. Disulfiram and diethyldithiocarbamate were purchased from Sigma Chemical Company (St. Louis,

Received for publication June 11, 1979.
[1] This work was supported by Grants HL15061 (SCOR) and HL23790 from the National Heart, Lung and Blood Institute.
[2] Established Investigator of the American Heart Association.

MO). Pluronic-F68 was obtained from BASF Wyandotte Corporation, Wyandotte, MI.

Rat exposures were done in a 40 liter hyperbaric chamber (model 1836-HP, Bethlehem Corporation, Bethlehem, PA) Five rats housed in individual cages were exposed at one time. The chamber was flushed with 100% O_2 until equilibrated and pressurized over a 5-min period to 2 atmospheres with a pressure regulator (Nullmatic model 40, Moore Products, Spring House, PA) and continuously vented through a small leak at a gas flow of 4 liters/min. The chamber atmosphere was monitored with an oxygen analyzer (Applied Electrochemistry, Inc., model S-3A, Sunnyvale, CA). Chamber CO_2 was kept below 0.5% with CO_2 absorbant and was monitored with an infrared CO_2 analyzer (model LB1, Beckman Instruments, Inc., Fullerton, CA). The temperature in the chamber was ~22° C, and relative humidity approached 100%. Rats were observed through a window at 4-hr intervals in order to determine survival.

SOD was measured in lungs of rats at either 1, 4 or 13 hr after injection of 200 mg/kg of drugs or vehicle and maintained in a normal atmosphere. The rats were anesthetized with pentobarbital (~50 mg/kg) and their lungs were perfused through the pulmonary artery with Krebs-Ringer phosphate buffer, pH 7.4, in order to remove blood. The postperfused tissue was then homogenized in Tris hydroxymethylaminomethane (0.025 M)-glycine (0.192 M) buffer, pH 8.3, with a Polytron (Kinematics GMBH, Luzern, Switzerland). Cytosolic SOD was assayed by its ability to inhibit the xanthine oxidase-catalyzed reduction of cytochrome *c* (McCord and Fridovich, 1969). Interference by cytochrome oxidase and mitochondrial SOD was eliminated by performing the assay at pH 10.2 (Crapo and McCord, 1976). The assay buffer contained in a 3-ml total volume, 0.05 M sodium bicarbonate, pH 10.2; 1.0×10^{-4} M EDTA; 1×10^{-5} M xanthine; and sufficient xanthine oxidase to produce an absorbance change at 550 nm of 0.025 unit/min in the absence of SOD.

The effects of disulfiram and DDC on cytosolic SOD activity *in vitro* were measured by incubating these inhibitors with 3275 units of cytosolic SOD (Diagnostic Data, Inc., Mountain View, CA) in 1 ml of 0.2 M potassium phosphate buffer, pH 7.8, for 1 hr at 37°C, containing 5% Pluronic-F68 (w/v) (to solubilize the disulfiram). Aliquots for each incubation were assayed for SOD activity as described above.

Statistical analysis of mortality data were based on the exact method χ^2 test, and the Student's *t* test for independent variables was used for comparisons of the SOD activities in lung tissue (Croxton, 1959).

Results

Disulfiram and DDC were tested for their effects on the mortality of rats exposed to hyperoxia. Figure 1 illustrates the observations at 4, 8, 12 and 16 hr of the exposed animals to 2 atmospheres absolute O_2. Both DDC and disulfiram at the same dose (200 mg/kg) decreased the survival time in oxygen at 2 atmospheres. The effect of DDC in shortening survival was significant compared with both control ($P < .01$) and disulfiram treatment ($P < .05$) after 8 hr of O_2 exposure. The effect of disulfiram was significant vs. control ($P < .05$) at 12 hr of exposure (fig. 1). The greatest observed difference in effect on mortality between DDC and disulfiram administration occurred at 12-hr exposure ($P < .02$). By 16 hr in hyperoxia, one of the 14 control rats, but all of the disulfiram- and DDC-treated rats, had died. Before death, rats were observed to have marked tachypnea and remained inactive, but no seizures or other manifestations of central nervous system toxicity were noted. None of the rats given DDC or disulfiram and kept in air at 1 atmosphere died within 13 hr postinjection.

The activity of cytosolic SOD in the lungs of rats kept in normoxia was measured at 1, 4 and 13 hr after injection of 200 mg/kg of DDC and 200 mg/kg of disulfiram or vehicle. Although both DDC and disulfiram on administration decreased

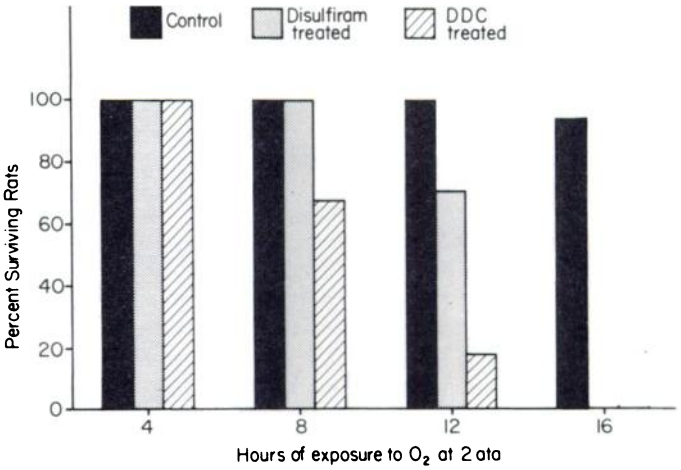

Fig. 1. Effect of disulfiram and DDC on survival time in hyperoxia. Adult rats were administered DDC (200 mg/kg; 12 rats) or disulfiram (200 mg/kg; 8 rats) as described in "Materials and Methods." Sixteen control rats received an equal volume of the suspended medium. One hour after injection, the animals were exposed to oxygen at 2 atmospheres absolute (ata).

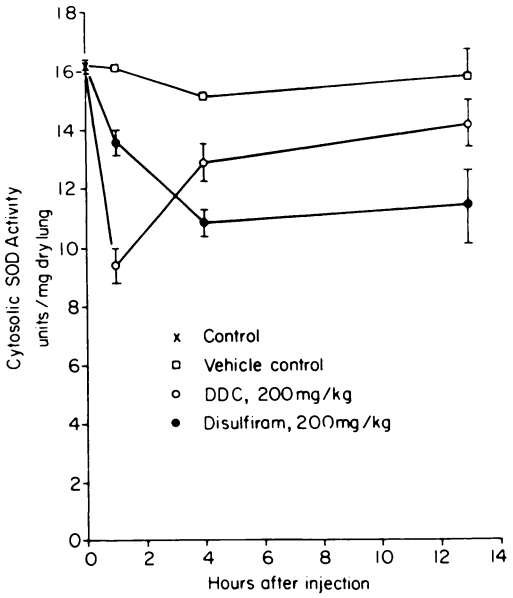

Fig. 2. Effect of disulfiram and DDC on pulmonary cytosolic SOD activity as a function of time postinjection. Rats were treated as described in "Materials and Methods." Zero time controls were not injected, although controls at other times were injected with the vehicle solution. Three rats were used for each determination except for controls (1 at 1 and 4 hr; 10 at 13 hr).

SOD activity, the time course of this effect by these compounds was different. Figure 2 shows that lung SOD activity after DDC administration was lowest at 1 hr (40.0 ± 3.6% inhibited; $P < .001$ *vs.* controls), had nearly returned to control level at 4 hr and was about the same as controls at 13 hr. On the other hand, disulfiram administration produced a greater decrease in SOD activity at 4 hr (30.6 ± 2.8%; $P < .001$ vs. controls) than at 1 hr ($P < .025$ *vs.* 4 hr), and the degree of inhibition at 13 hr was essentially unchanged from that at 4 hr. DDC caused a significantly greater loss of activity at 1 hr (40.3 ± 3.6%) than did disulfiram (16.2 ± 2.7%; $P < .01$ disulfiram *vs.* DDC), although the effects of disulfiram appeared to persist longer than that of DDC.

The *in vitro* effects of DDC and disulfiram on purified cytosolic SOD are shown in table 1. Incubation with DDC caused marked inactivation of SOD *in vitro*, although disulfiram at the same concentration had no effect.

Discussion

In this study, rats were exposed to 2 atmospheres absolute O_2. Exposure at this pressure which was below the seizure threshold had the advantage of accelerating pulmonary damage thereby minimizing the risk of potential complications such as infection and starvation that might occur with longer term exposure at lower O_2 partial pressures. Histological studies made in this laboratory and reported elsewhere (Fisher *et al.*, 1979) have shown that rats subjected to 2 atmospheres absolute O_2 near the average time of death (16 to 20 hr) had evident pulmonary damage but no obvious neurological manifestations of oxygen toxicity.

The administration of either DDC or disulfiram to rats caused a decrease in their survival on exposure to O_2 at 2 atmospheres absolute (fig. 1). Survival in varying O_2 pressures appears to depend to a great extent on the levels of SOD activity as demonstrated in several species of microorganisms (Fridovich, 1976). Increased SOD in the lungs of rats has been correlated with increased resistance to death from pulmonary oxygen toxicity (Crapo and Tierney, 1974). Frank *et al.* (1978) have demonstrated that decreased pulmonary SOD due to administration of DDC correlated with decreased survival time in 95 to 97% O_2 at 1 atmosphere. In this study we have shown that DDC had a much greater effect on mortality at 2 atmospheres absolute O_2 than was reported at 1 atmosphere absolute O_2 by Frank *et al.* (1978) and Deneke *et al.* (1979). We also found that 200 mg/kg of DDC was more lethal than an equal dose of disulfiram in 2 atmospheres absolute O_2 which was the opposite of the previously reported relative effects of these compounds at 1 atmosphere absolute O_2 (Deneke *et al.*, 1979).

In its use as an adjunct in treatment of chronic alcoholism, disulfiram has been found to require 12 hr for its peak effectiveness and then is slowly eliminated, probably due to its high fat solubility and accumulation in fat depots (Ritchie, 1975). DDC, however, has previously been shown to have a relatively short effectiveness *in vivo* as its peak inhibition of SOD activity in liver, brain and lung is within the first hr after administration (Heikkila *et al.*, 1976; Frank *et al.*, 1978). It seems reasonable that the observed difference in the pharmacokinetics of DDC and disulfiram (fig. 2) is partially a result of the relatively higher lipid-aqueous solubility of disulfiram.

It is well established that disulfiram is converted *in vivo* to DDC in all organs examined, although lungs have not yet been studied (Stromme, 1965; De Saint Blanquat *et al.*, 1976). In agreement with Heikkila *et al.* (1976), we demonstrated that DDC inhibits cytosolic SOD *in vitro*. On the other hand, disulfiram *in vitro* did not inhibit cytosolic SOD (table I). Thus, the difference between *in vivo* and *in vitro* effects of disulfiram is probably due to its metabolic conversion *in vivo* to the SOD inhibitor DDC. Subsequent inhibition of SOD after conversion to DDC is probably the mechanism for the potentiation of oxygen toxicity by disulfiram.

We can make a rough comparison of the *in vitro* and *in vivo* effects of DDC concentrations on SOD activity. If the dose of DDC (200 mg/kg) was evenly distributed throughout the total body water the concentration would have been $\sim 2 \times 10^{-3}$ M which was in the range of DDC concentrations which produced significant SOD inactivation *in vitro*.

Deneke *et al.* (1979) showed that SOD activity in the lungs was decreased by both disulfiram and DDC at 4 hr but had recovered by 24 hr. Because of this recovery of activity and the apparent greater potentiation of O_2 toxicity by disulfiram, these authors concluded that enhancement of O_2 toxicity by disulfiram was not due to SOD inhibition through conversion to DDC. What they did not consider was that the effect of DDC in decreasing SOD activity could be more transient than for disulfiram. Secondly, an increased rate of organ damage during the period in which SOD was inhibited could contribute to the accumulated damage even if SOD activity itself recovered. During the relatively long period necessary for death due to 1 atmosphere absolute O_2 exposure [average time to death of controls was 48 to 72 hr (Deneke *et al.*, 1979)], the damage due to the transient effect of DDC could have been repaired, although the longer lasting effect of disulfiram was observed. At 2 atmospheres absolute O_2, the effect of SOD inhibition by DDC would have been much more significant because the faster rate of accumulation of damage at the higher PO_2 considerably shortens the time to death [average time to death of controls $\approx$18 hr (Fisher *et al.*, 1979)]. Because DDC was more potent as an SOD inhibitor than disulfiram during this shorter period, it probably caused a greater enhancement of O_2 damage. Our studies do not contradict the findings of Deneke *et al.* (1979) but add new information which is consistent with the opposite conclusion.

Since DDC and disulfiram are structurally similar and metabolically interconvertible, a consideration of chemistry of these compounds in relation to their effects on enzymes may provide an understanding of the reasons for the differences in their *in vivo vs. in vitro* effects. Cytosolic SOD is a copper containing enzyme and DDC inhibition can be reversed with added copper (Heikkila *et al.*, 1976). Although DDC and disulfiram both form complexes with copper, the active site of copper-containing enzymes would probably be more accessible to the smaller and hydrophyllic DDC. Studies with the essential copper-containing enzyme, dopamine-β-hydroxylase, demonstrates this difference. DDC inhibits this enzyme *in vitro*, but disulfiram does not unless a reductant is present (Goldstein *et al.*, 1964). However, the opposite effect of disulfiram and DDC on *in vitro* enzyme inhibition has also been demonstrated. Disulfiram is able to function directly in disulfide exchange reactions, whereas DDC requires an oxidant to do so. *In vitro* inhibition of the essential sulfhydryl-containing enzyme, hexokinase, by disulfiram but not DDC (unless an oxidant is present) can therefore be understood (Stromme, 1963). The sulfhydryl

TABLE 1

Effect of disulfiram and DDC *in vitro* on cytosolic SOD activity

Separate incubations were carried out in the presence of the indicated concentrations of DDC or disulfiram or without added inhibitors (control). Results are the mean ± S.E. for four incubations at each concentration. For each inhibitor, the value obtained in the presence of the inhibitor was compared with control.

	SOD Activity	% Control Activity
	units/ml	
Control	3275 ± 134	
Disulfiram		
10^{-4} M	3304 ± 64	101 ± 2
10^{-3} M	3391 ± 78	104 ± 2
DDC		
10^{-4} M	2692 ± 128	82 ± 4
10^{-3} M	1401 ± 135	43 ± 4

groups of cytosolic SOD appear to be unreactive (Forman *et al.*, 1973) so that it is not surprising that disulfiram does not inhibit this enzyme *in vitro*. Thus, *in vivo* reduction of disulfiram to DDC (a copper chelator) probably accounts for the decrease of cytosolic SOD (a copper-containing enzyme) caused by disulfiram administration.

Both disulfiram as well as DDC have been reported to protect mice against seizures in hyperbaric oxygen (Gershman *et al.*, 1958; Faiman *et al.*, 1971). The mechanism involved in seizure prevention by DDC and disulfiram in short-term exposure to 4 or more atmospheres of O_2 is unclear. The present studies indicate that these compounds administered to rats in conjunction with prolonged oxygen exposure below the seizure threshold shortens the time to oxygen toxicity. This dichtomy of effect illustrates the pitfall of extrapolating the effect of agents on the convulsive threshold to include pulmonary manifestations of toxicity.

References

Clark, J. M. and Lambertsen, C. J.: Pulmonary oxygen toxicity: A review. Pharmacol. Rev. **23:** 37-133, 1971.

Crapo, J. D. and McCord, J. M.: Oxygen induced changes in pulmonary superoxide dismutase assayed by antibody titrations. Am. J. Physiol. **231:** 1196-1203, 1976.

Crapo, J. D. and Tierney, D. F.: Superoxide dismutase and pulmonary oxygen toxicity. Am. J. Physiol. **226:** 1401-1407, 1974.

Croxton, F. E.: Elementary Statistics, pp. 235-239, 275-276, Dover, 1959, New York.

Deneke, S. M., Bernstein, S. P. and Fanburg, B. L.: Enhancement by disulfiram (Antabuse) of toxic effects of 95 to 97% O_2 on the rat lung. J. Pharmacol. Exp. Ther. **208:** 377-380, 1979.

DeSaint Blanquat, Vidaillac, G., Lindenbaum, A., and Derache, R.: Absorption digestive, fixation tissulaire et excretion du disulfirame administre oralement chez le rat. Arch. Int. Pharmacodyn. Ther. **223:** 339-350, 1976.

Faiman, M. D., Mehl, R. G. and Oehme, F. W.: Protection with disulfiram from central and pulmonary oxygen toxicity. Biochem. Pharmacol. **20:** 3059-3067, 1971.

Faiman, M. D., Nolan, R. J. and Oehme, F. W.: Effect of disulfiram on oxygen toxicity in Beagle dogs. Aersp. Med. **45:** 29-32, 1974.

Fisher, A. B., Kravetz, G. and Forman, H. J.: Induction of tolerance to hyperbaric oxygen in rats by oxygen pre-exposure. Am. Rev. Resp. Dis. 1980.

Forman, H. J., Evans, H. J., Hill, R. L. and Fridovich, I.: Histidine at the active site of superoxide dismutase. Biochemistry **12:** 823-827, 1973.

Frank, L., Wood, D. L. and Roberts, R. J.: Effect of diethyldithiocarbamate on oxygen toxicity and lung enzyme activity in immature and adult rats. Biochem. Pharamcol. **27:** 251-254, 1978.

Fridovich, I.: Oxygen radicals, hydrogen peroxide and oxygen toxicity. *In* Free Radicals in Biology, ed. by W. A. Pryor, pp. 239-277, Academic Press, New York, 1976.

Gerschman, R., Gilbert, D. L. and Caccamise, D.: Effects of various substances on survival times of mice exposed to different high oxygen tensions. Am. J. Physiol. **192:** 563-571, 1958.

Goldstein, M., Anagnoste, B., Lauber, E. and McKereghan, M. R.: Inhibition of dopamine-β-hydroxylase by disulfiram. Life Sci. **3:** 763-767, 1964.

Hald, J., Jacobsen, E. and Larsen, V.: The sensitizing effect of tetraethylthiuramdisulphide (Antabuse) to ethylalcohol. Acta Pharmacol. Toxicol. **4:** 285-296, 1948.

Heikkila, R. E., Cabbat, F. S. and Cohen, G.: *In vivo* inhibition of superoxide dismutase in mice by diethylthiocarbamate. J. Biol. Chem. **251:** 2182-2185, 1976.

McCord, J. M. and Fridovich, I.: Superoxide dismutase. J. Biol. Chem. **244:** 6049-6055, 1969.

Ritchie, J. M.: The aliphatic alcohols. *In* The Pharmacological Basis of Therapeutics, 5th ed., ed. by L. S. Goodman and A. Gilman, pp. 148-149, MacMillan Publishing, New York, 1975.

Stromme, J. H.: Inhibition of hexokinase by disulfiram and diethyldithiocarbamate. Biochem. Pharmacol. **12:** 157-166, 1963.

Stromme, J. H.: Metabolism of disulfiram and diethyldithiocarbamate in rats with demonstration of an *in vivo* ethanol induced inhibition of the glucuronic acid conjugation of the thiol. Biochem. Pharmacol. **14:** 393-410, 1965.

Send reprint requests to: Dr. H. J. Forman, Department of Physiology/G4, University of Pennsylvania, Philadelphia, PA 19104.