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Effect of diethyldithiocarbamate on oxygen toxicity and lung enzyme activity in immature and adult rats *

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Increased tolerance to hyperoxia of the immature animal compared to that of the adult is well documented, though the explanation for this phenomenon is not known [1–4]. We have previously reported that there is a rapid increase in superoxide dismutase (SOD) activity in the immature animal lung on exposure to high O_2 concentrations both *in vitro* and *in vivo* [5–7]. SOD is an enzyme present in all aerobic organisms and functions as a scavenger of superoxide anion (O_2^-), a highly reactive and cytotoxic free radical reduction product of molecular O_2 ($2H^+ + 2O_2^- \xrightarrow{SOD} O_2 + H_2O_2$). Since the intracellular production of O_2^- is increased under conditions of hyperoxia [8, 9], the ability to effectively increase SOD activity in response to a hyperoxic challenge may serve an important cellular protective function. Many studies in prokaryotes have established an association between the increased resistance of an organism to O_2 toxicity and the ability of the organism to increase its enzyme complement of SOD [10–12].

Recently, Heikkila *et al.* [13] reported on the SOD-inhibiting effect *in vivo* of the Cu-chelating agent, diethyldithiocarbamate (DDC). After preliminary time–response and dose–response studies with DDC, we chose to use this agent to try to further elucidate the importance of SOD as a protective enzyme in eukaryotes during exposure to high concentrations of O_2. We also measured the effect of DDC treatment on another Cu-containing enzyme which functions in intracellular O_2 handling, cytochrome oxidase.

Animals used in this study were Sprague–Dawley albino rats bred and raised in the Animal Care Facilities at The University of Iowa. The adult rats used weighed between 250 and 300 g, the 20- to 33-day-old rats between 50 and 90 g, and the 3- to 5-day-old rats between 10 and 15 g. Diethyldithiocarbamate (Sigma Chemical Co., St. Louis, MO) for injection i.p. was prepared fresh daily in 0.9% NaCl. To reduce the irritating effect of the injections, the pH of the solution was adjusted to 7.4–8.0 with a few drops of 1.0 N HCl.

For the preliminary time–response experiments, young and adult rats were given 0.75 g/kg, i.p., of DDC and sacrificed at 1, 3, 6, 12, 18 and 24 hr post-injection. For preliminary dose–response experiments, rats received DDC doses ranging from 0.25 to 3.0 g/kg and were sacrificed at 3 hr post-injection. Control rats in all the experiments received equi-volume injections of saline. For the hyperoxic studies, rats were exposed continuously in stainless steel exposure chambers to 21% or 95–97% O_2 for 48 hr, with a 15- to 30-min interval out of the chambers at 24 hr to facilitate cage cleaning and injections. Food and water were provided *ad lib.* Oxygen concentration was continuously monitored with a Beckman model OM-11 gas analyzer. Carbon dioxide levels, monitored with a Beckman model LB-2 gas analyzer, remained below 0.4 per cent in all experiments. The environmental temperature and vapor pressure in the exposure chambers were maintained between 24 and 26° and less than 10 mmHg respectively. For all the young rat experiments, litters were randomized prior to the start of the procedures. The young rats were kept with mother rats, and mother rats were exchanged between the air- and high O_2-exposed rat pups at 24 hr.

At the end of each experiment, the rats were sacrificed by decapitation, and the lungs were removed immediately, and placed in ice-cold isotonic buffer (0.1 M potassium phosphate, 0.15 M KCl, pH 7.4). The lungs were then pressed dry between gauze pads and weighed prior to homogenization for 2 min in 0.005 M potassium phosphate buffer, pH 7.8, at 5° with a Sorvall Omnimixer. A dilution factor of 1:15 (w:v) was used for homogenization. SOD activity was measured in the fresh samples according to the standard method of McCord and Fridovich [14] based on the inhibition of ferricytochrome *c* reduction by xanthine and xanthine oxidase, with one unit of activity

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Table 1. Dose–response: effects of DDC treatment on survival and lung enzymes in young and adult rats*

DDC dose	Adult rats			20–25 days			3–5 days		
	Survival	SOD	Cyto. oxid.	Survival	SOD	Cyto. oxid.	Survival	SOD	Cyto. oxid.
0	12/12 (100)	100.0	100.0	14/14 (100)	100.0	100.0	14/14 (100)	100.0	100.0
0.25	12/12 (100)	96.3	97.7	12/12 (100)	97.1	92.7	13/14 (93)	99.0	99.9
0.5	12/12 (100)	84.0	95.1	12/12 (100)	87.9	94.4	13/14 (93)	93.0	90.5
0.75	32/32 (100)	—	—	24/24 (100)	—	-	33/36 (92)	—	—
1.0	11/12 (92)	72.7	90.7	10/12 (83)	81.2	86.4	10/14 (71)	82.0	93.0
1.5	1/12 (8)	69.3	84.7	4/12 (33)	74.2	85.1	3/14 (21)	70.5	89.5
2.0	0/12 (0)	59.4	74.2	0/13 (0)	67.5	84.0	0/14 (0)	63.5	80.5
3.0	1/12 (8)	52.4	71.6	0/13 (0)	58.9	71.0	0/14 (0)	58.5	79.0

* Results of two or three experiments in each age group. Rats were injected with 0.25 to 3.0 g/kg of DDC and sacrificed 3 hr post-injection. Control received saline, i.p. SOD and cytochrome oxidase activity are expressed as per cent of control value. (Control values for SOD and cytochrome oxidase in adults, 20–25 day and 3–5 day rats were, respectively: 1324 ± 52 units/g of lung and 5342 ± 108 nmoles cytochrome *c* oxidized/g of lung; 802 ± 93 and 3732 ± 118; 948 ± 3 and 3905 ± 72.) Values in parentheses = per cent survival.

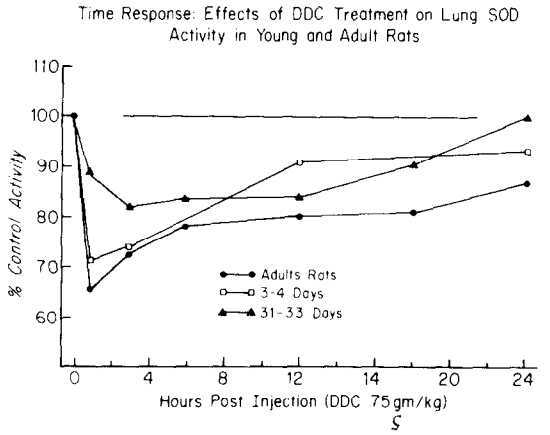

Fig. 1. Results of repeat experiments using 4–5 adults, and 5–6 (31- to 33-day-old) young rats and 6–8 (3- to 4-day-old) immature rats sacrificed at each time interval. SOD activity was calculated on the basis of units/g of lung tissue and expressed as per cent of control value. DDC dose = 0.75 g/kg, i.p., at zero time.

defined as the amount of sample required to inhibit the reference reaction rate by 50 per cent. The reaction mixture for this analysis contained cytochrome *c* (5×10^{-4} M), 0.03 cc xanthine (1×10^{-2} M), 1.4 cc distilled water, and 1.5 cc phosphate buffer (0.1 M with 2×10^{-4} M EDTA, pH 7.8). Absorbance was followed at 550 nm after the addition of enough xanthine oxidase (50 units/ml) to give a reference reaction rate of 0.02500 A units/min. Cytochrome *c*, type VI, xanthine and xanthine oxidase were obtained from Sigma Chemical Co. Cytochrome oxidase activity was measured by the method of Wharton and Tzagoloff [15]. The reaction mixture consisted of 0.20 ml reduced cytochrome *c* plus 2.80 ml of 0.01 M phosphate buffer, pH 7.0. The reaction was initiated by the addition of 25–30 μl homogenate. Activity units were expressed as nmoles cytochrome *c* oxidized/g of lung. A Varian Techtron spectrophotometer model 635 was employed for quantitation of the assays. DNA assays were done by the method of Richards [16]. SOD activity was expressed both as units/g of lung tissue and units/mg of DNA. Expressed as per cent of control enzyme values, these results were similar. For the purpose of this paper, units/g of lung are reported, since DNA values were not performed on all samples.

The results of dose–response studies (DDC, 0.25 to 3.0 g/kg) in several groups of young and adult rats are shown in Table 1. There was a sharp decline in survival of each age group between the 1.0 and 1.5 g/kg dosage level. The calculated LD_{50} values were 1.23 (adults), 1.30 (20–25 days), and 1.08 g/kg (3–5 days). Increasing inhibition of pulmonary SOD and cytochrome oxidase activity was observed with increasing dosages of DDC. At a dose of 1.5 m/kg, the average SOD activity decrease was approximately 29 per cent in the rat lungs. (This compares to a 48 per cent decrease in brain and a 71 per cent decrease in liver reported by Heikkila *et al.* [13] for this same dosage level in the mouse.) At the maximal dosage used (3.0 g/kg), the decrease in SOD activity compared to control lungs averaged approximately 43 per cent and was rather similar in all age rats tested (Table 1). The lung cytochrome oxidase activity inhibition at this dosage was approximately 28 per cent. Time–response studies for inhibition of lung SOD activity with DDC treatment were performed, using a dose of 0.75 g/kg (Fig. 1). In these experiments, adults and 3–4 day rats showed a maximal decrease in lung SOD activity at 1 hr post-injection (−34 and −29 per cent, respectively) with a gradual return of activity over the ensuing 24-hr experimental period. At 24 hr adult lung SOD was still 13 per cent less than control values, while 3–4 day lungs had only 6 per cent less than control activity. The 31- to 33-day-old rats showed a maximal inhibition at 3 hr post-injection (−18 per cent) and a return to control levels by 24 hr.

For the O_2-exposure experiments, rats aged 3–5 days from several litters were randomized and injected daily with either DDC (0.75, 0.50, 0.25 or 0.125 g/kg) or saline and housed in either room air or 95–97% O_2 in the exposure chambers. The 0.75 g/kg dose of DDC proved too toxic on daily administration to both the O_2- and air-exposed animals (Fig. 2). With the 0.5 and 0.25 g/kg doses of DDC, there was a significant difference in lethality observed between the air- and O_2-exposed animals after 48 hr of exposure. The 0.125 g/kg dosage of DDC was found to be too low to provide lung SOD enzyme inhibition by 24 hr after administration, and did not affect the survival of the O_2–air-exposed rats. Figure 3 shows the average pulmonary enzyme changes in groups of 3–5 day rats given DDC (0.5 or 0.25 g/kg) at 0 and 24 hr and sacrificed after 48 hr of air or hyperoxia exposure. To be noted in the SOD results is that all the control animals survived in O_2 or air, and the O_2-exposed controls (saline injected) all demonstrated increased pulmonary SOD activity compared to the air-exposed controls. Also, the air-exposed

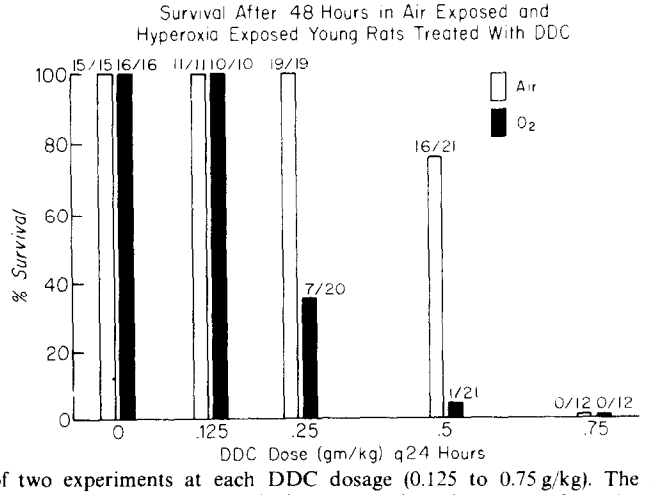

Fig. 2. Results of two experiments at each DDC dosage (0.125 to 0.75 g/kg). The 3- to 5-day-old rats received DDC at 0 and 24 hr and survival was recorded after 48 hr of continuous exposure to 21% or 95–97% O_2.

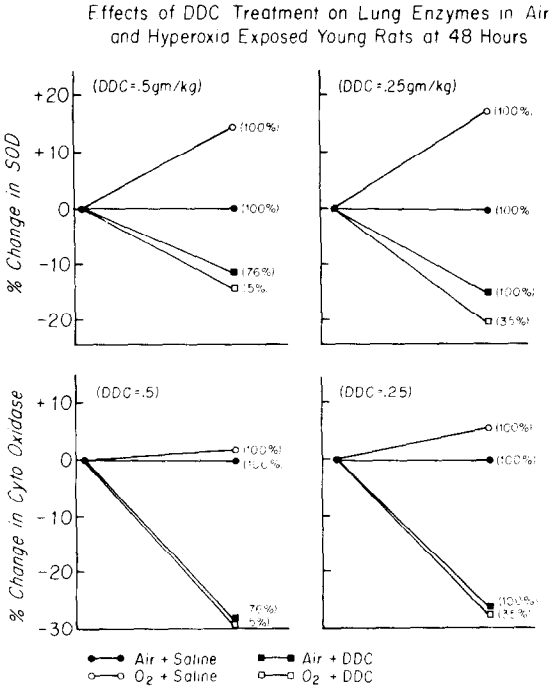

Fig. 3. Results of two experiments at each DDC dosage level (0.25 and 0.50 g/kg). Rats were given DDC, i.p., at 0 and 24 hr and sacrificed at 48 hr after continuous exposure to air or 95–97% O_2. SOD and cytochrome oxidase activity were calculated as units/g of lung and nmoles cytochrome *c* oxidized/g of lung, respectively, and expressed as per cent change in enzyme activity compared to air-exposed controls (saline, i.p.). Values in parentheses = per cent survival.

and O_2-exposed rats treated with DDC had significantly different survival at both dosages employed (air totals: 35/40; O_2 totals: 8/41) ($P < 0.01$). While producing an equal degree of inhibition of basal lung SOD activity in the air- and O_2-exposed animals, the DDC also prevented the expected increase in SOD activity normally observed in young rats exposed to hyperoxia. In this same series of experiments (Fig. 3), O_2 exposure did not result in an increased basal activity of lung cytochrome oxidase. We have previously observed a lack of a cytochrome oxidase response to hyperoxia in many experiments in young rats in which an SOD response was observed (unpublished data). DDC treatment produced a comparable decrease in cytochrome oxidase activity in the air- and O_2-exposed animals.

We have previously reported that young rats tolerate 95 + % O_2 for at least 5 days without gross evidence of pulmonary damage and their hyperoxic tolerance is accompanied by an increase in pulmonary SOD activity [7]. Adult rats, in contrast, all show gross evidence of pulmonary edema after 48–72 hr of similar O_2 exposure and 70 per cent die during this hyperoxic challenge. The lung enzymes of the glutathione system (GSH, GSH-peroxidase, GSH-reductase, and glucose 6-phosphate dehydrogenase) also show increased activity in young rats exposed to hyperoxia, and the augmented activity of both of these antioxidant enzyme defense systems (SOD and GSH) may help to explain biochemically the marked tolerance of immature rats to hyperoxia compared to adults, whose lung SOD and GSH enzyme activities do not increase during hyperoxic exposure [7].

DDC, by inhibiting the Cu-containing form of SOD in the cytosol (mitochondrial SOD is a mangano-enzyme) may be a useful pharmacological tool in further establishing the vital protective role of this enzyme in the lung challenged with hyperoxia. With DDC preventing the normally rapid increase in SOD activity from occurring in the O_2-exposed young rats, the 48-hr survival in hyperoxia fell from 100 per cent (42/42) to 21 per cent (8/41) ($P < 0.01$). DDC toxicity itself does not explain this marked alteration in O_2 tolerance, since the air controls treated with DDC nearly all survived (35/40). DDC treatment in effect converted the lung SOD response to hyperoxia of the immature rats to that of the adult rat who is intolerant of prolonged high O_2 exposure.

We also chose to study the effect of DDC on another Cu-containing enzyme involved in O_2 metabolism and cellular respiration, cytochrome oxidase. DDC, as a Cu-chelator, has been previously used to inhibit certain other Cu-containing enzymes including aldehyde dehydrogenase in the liver [17] and dopamine-β-hydroxylase in adrenergic nervous tissue [18], but not, to our knowledge, lung cytochrome oxidase. We found that in the dosage range used to inhibit SOD activity in the rat lung, a concurrent inhibition of cytochrome oxidase was observed in all age rats studied. The possible importance of a diminished level of activity of cytochrome oxidase and its possible toxic consequences in the lung exposed to the increased intracellular O_2 flux of hyperoxic exposure are not known. However, the basal levels of this enzyme do not normally show any rapid increase in activity on hyperoxic exposure, as is normally seen with SOD in the lungs of O_2 tolerant young animals.

A better understanding of the pharmacology of DDC [mechanism(s) of its toxicity and the role of its inhibitory effect on other enzymes] will enhance its value in future biochemical investigations of O_2 toxicity. In particular, it will be important to determine whether hyperoxia and DDC have a synergistic inhibitory effect on the activity of various lung sulfhydryl-containing enzymes, since high O_2 levels and DDC are known to have such inhibitory effects independently [19,20]. Until then or until a more selective inhibitor of SOD is available, we can only suggest from our studies that a positive association does exist between decreased SOD activity with DDC treatment and decreased O_2 tolerance in young animals normally tolerant to hyperoxia.

Addendum—Two previous reports of the use of DDC to alter O_2 toxicity have been discovered [21,22]. However, the authors of these reports used DDC in an attempt to protect animals from the rapid central nervous system toxicity of hyperbaric O_2 (4–6 atms) and no pulmonary studies were performed. The reports, incidentally, gave contradictory findings—one showed a protective effect for DDC [21] and the other a detrimental effect [22].

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Studies on the inhibition of phosphodiesterase-catalyzed cyclic AMP and cyclic GMP breakdown and relaxation of canine tracheal smooth muscle

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Several pharmacological agents are known which inhibit cyclic 3′,5′-nucleotide phosphodiesterase(s)(EC 3.1.4.17) and also produce bronchodilation [1–7]. The present investigation was undertaken to determine whether a quantitative correlation could be found in respiratory smooth muscle between the phosphodiesterase (PDE) inhibition and muscular relaxation produced by some of these agents. Our findings support the concept [8, 9] that relaxation is produced as a result of pharmacological inhibition of cyclic-3′,5′-adenosine monophosphate (cAMP) breakdown and the subsequent accumulation of cAMP in cells. Inhibition of cyclic-3′,5′-guanosine monophosphate (cGMP), a tissue constituent that has been implicated as a stimulator of smooth muscle contraction, can also be correlated with relaxation, although in most cases inhibition of cGMP is not as great as for cAMP.

Theophylline, caffeine, acetyl-β-methylcholine Cl (methacholine), *l*-isoproterenol-*d*-bitartrate, cAMP and

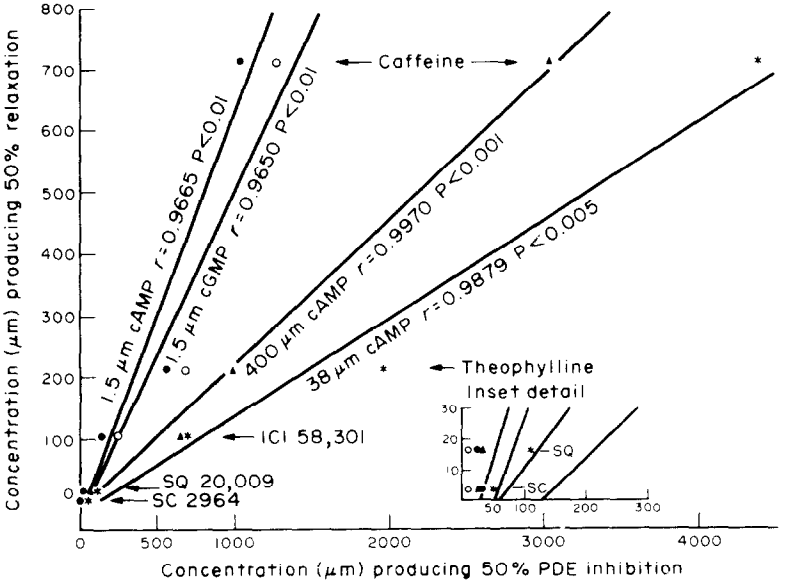

Fig. 1. Correlations between drug concentrations producing 50 per cent tracheal smooth muscle relaxation vs concentrations producing 50 per cent inhibition of phosphodiesterase under four different substrate conditions. At the 50 per cent relaxing concentration indicated for each drug are four points corresponding to the 50 per cent PDE-inhibiting concentrations of the same drug with 1.5 μM cGMP (○), or 1.5 (●), 38(*) or 400 (▲) μM cAMP as substrate. Data represent geometric means derived from four to seven relaxation curves or three to four inhibition experiments and calculated after Fleming *et al.* [12]. Standard errors ranged from 1.1 to 2.1 μM. Regression lines were drawn by the method of least squares. P indicates the probability that the linear correlation coefficient, *r*, is zero. Points clustered in the lower left corner are shown in greater detail in the inset.