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Experimental data on the prevention of retrolental fibroplasia by D-penicillamine

by

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Superoxide dismutase activity of alveolar macrophages, lung, liver, retina and choroid has been studied in rats and rabbits exposed to oxygen at high concentration by itself and in combination with D-penicillamine. Prolonged hyperoxia caused an increase in superoxide dismutase activity while D-penicillamine was found to inhibit the inductive effect of oxygen. It is assumed that the antioxidative effect of D-penicillamine makes it unnecessary to augment the production of superoxide dismutase.

In previous investigations into the mechanism of phagocytosis, the production and importance of free oxygen radicals present in the cells during the bactericidal phase were studied. The free radicals are produced from molecular oxygen and they contain unpaired electrons of great reactivity [4, 12, 13]. During phagocytosis a respiratory burst reaction develops when the rate of cellular oxygen consumption and glycolysis increases. The result of these processes is the production of oxygen radicals: superoxide anion (O_2^-), peroxide radical (O_2^{2-}), hydroxyl radical ($OH\cdot$) and the singlet oxygen ($^1O_2$) [2, 3, 8, 10, 15, 20, 27, 31, 32]. The question arises how far can the cells of normal newborn, and particularly of premature, babies meet this increased metabolic demand.

Das et al [7] found that the monocytes of newborns functioned with an approximately 25 to 30% energy deficit, since the amount and/or the activity of pyruvate kinase in these cells is significantly reduced. This enzyme is responsible for the glycolytic ATP production in the third step of glycolysis. It was therefore of interest to clarify whether the enzymes playing a role in the inactivation of free radicals produced in the process of phagocytosis do really meet the extra demands. First of all we investigated the activity of the enzyme superoxide dismutase (SOD) which catalyzes the transformation of superoxide anion (O_2^-), the first radical produced from molecular oxygen, to hydrogen peroxide and oxygen (10). SOD is a metallo-protein enzyme which has several names according to the place of isolation: hematocuprein, hepatocuprein, cerebrocuprein, etc. According to Johnston [15] SOD accelerates the rate of the reaction

$O_2^- + O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$, by a factor of 10 000. Its active components are copper and zinc. Copper exerts the oxido-reductive function; Cu^{II} oxidizes the substrate, and Cu^{I} is immediately reduced to Cu^{II} by oxygen [36]. The methods of its activity assay [27, etc.] are based on the fact that SOD inhibits the spontaneous oxidation of epinephrine to adrenochrome, and consequently reduces the colour intensity produced by adrenochrome at pH 10.2 in the presence of air.

During phagocytosis the micro- and macrophages increase their oxygen consumption. This mechanism is of primary importance for cellular energy metabolism and for bactericidal activity but the negative effect of the remaining free radicals must also be taken into account, especially in view of their assumed involvement in the results of postnatal oxygen therapy and also in its irreversible sequelae, retrolental fibroplasia (RLF) and bronchopulmonary dysplasia.

As to the frequency of RLF, indirect ophthalmoscopy revealed 11 cases among 407 children who had received oxygen therapy in the perinatal period. Of these, only one single baby out of 158 developed RLF before the introduction of intensive perinatal care. The condition occurred only in babies whose birth weight was less than 1500 g. The proportion of RLF was significantly improved by D-penicillamine (DPA), a compound which we have used with great success in cases of neonatal jaundice [22, 23]. Under its effect the occurrence of RLF fell from 10 : 289 to 1 : 118 in the whole material, and from 10 : 132 to 1 : 61 in the prematures under 1500 g birthweight.

In view of the above experience, animal experiments were performed to study the effect on the SOD activity of alveolar macrophages of prolonged oxygen and combined oxygen plus DPA treatment, further the changes in SOD activity under hyperoxic conditions in the neonatal period, and the effect of DPA treatment on the protective mechanism.

Material and Methods

The first question was studied on 7-day-old suckling rabbits, and the second one on 3—4-day-old Wistar rats, kept together with their mother under the usual circumstances. Four experimental groups were set up. The animals in *Group 1*, 10 rabbits and 23 rats, served as control and received only physiological salt solution. Animals in *Group 2*, 10 rabbits and 21 rats, were injected intraperitoneally with 600 mg/kg of DPA (Metalcaptase^R, Knoll AG., Ludwigshafen) twice during the experiment. Animals in *Group 3*, 11 rabbits and 27 rats, were placed for 36 hours into a baby incubator at constant 24°C, where oxygen concentration was kept between 85 and 90%. Oxygen concentration was tested by a Medicor type IK-13/A detector every 2 hours during the day and every 4 hours during the night. Animals in *Group 4*, 9 rabbits and 28 rats, were exposed to hyperoxia as in Group 3, and treated with DPA twice during the experiment, as in Group 2.

After sacrifice by decapitation the organs were immediately excised and washed blood-free in physiological salt solution. Alveolar macrophages were obtained from the rabbits by tracheobronchial flushing through a canula. The solution

was centrifuged at 200 *g* for 10 minutes and sonicated. From each animal lung and liver extract was gained by homogenization in a Porter-Elvehjem apparatus, and the extract was centrifuged as described above.

Retina and choroid tissue were isolated from both eyes under the light microscope and prepared as above. Due to the small amount of tissue, one sample consisted of the pooled tissues of three animals.

Determination of SOD activity in the supernatant was performed according to Misra and Fridovich [27], De Chatelet et al [8] and Novak et al [30]. One enzyme unit (U) was characterized by the activity which caused a 50% inhibition in the rate of epinephrine → adrenochrome transformation. The reaction was followed by a Beckman Acta CV spectrophotometer at 480 nm.

L-epinephrine was used at a concentration of 10 mM in 0.1 N HCl. In the case of spontaneous transformation, when no sample was added to the incubation medium, 100 μl was consumed from the stock solution in the presence of 2.9 ml sodium bicarbonate — HCl buffer pH 10.2 and 0.2 mM EDTA. In the course of measuring the enzyme activity, we added a 50% inhibition producing amount of the sample into the buffer solution. SOD activity was expressed as U per 10^6 alveolar macrophages per ml washing solution, or per 1 g tissue weight. The kinetics of autooxidation was followed for 3 to 5 minutes and delta-U was measured from the steepest portion of the reaction curve.

Statistical analysis of the results was done by Student's unpaired *t*-test.

Results

Figure 1 shows the SOD activity of alveolar macrophages. The activity increased by about 25% in the animals exposed to high oxygen concentration, and this was not significantly influenced by the simultaneous addition of DPA. In Figure 2 is seen the SOD activity in the lung. It was similar to that described above except that DPA treatment significantly decreased the hyperoxia induced increase of enzyme activity. Figure 3 demonstrates SOD activity in the lung, liver and retina-choroid in the untreated rats. SOD activity was highest in the liver, one third of this in the lung and the lowest

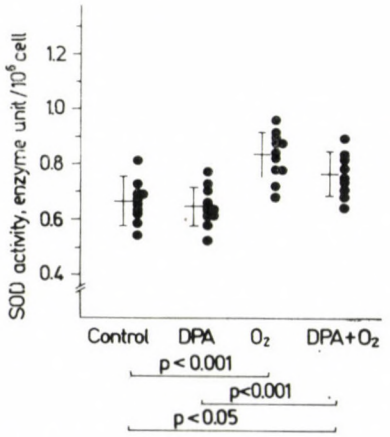

Fig. 1. Superoxide dismutase activity in alveolar macrophages of the newborn rabbit, after D-penicillamine (DPA), oxygen (O_2) and combined DPA + O_2 treatment

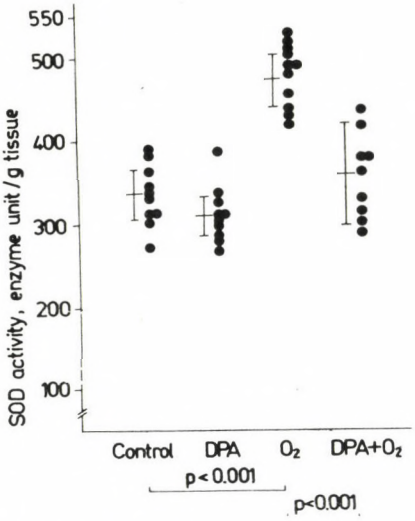

FIG. 2. Superoxide dismutase activity in the lung of newborn rabbits after D-penicillamine (DPA), oxygen (O_2) and combined DPA + O_2 treatment

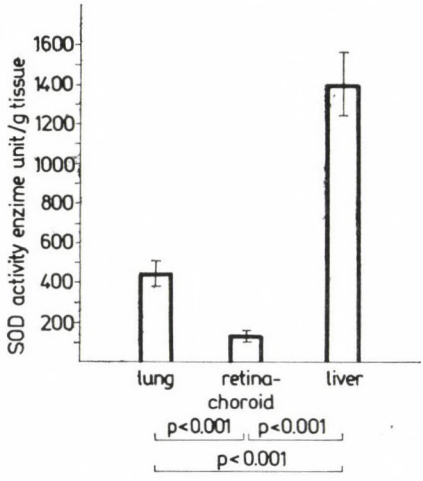

FIG. 3. Normal superoxide dismutase (SOD) activity in enzyme units/g tissue weight in the lung, retina-choroid and liver of newborn rats

in the retina and choroid. SOD activity of the rat lung extract was significantly less after oxygen plus DPA treatment than the latter was after oxygen treatment alone, but still higher than the value obtained in the control animals (Figure 4).

SOD activity of the retina-choroid was slightly reduced by DPA treatment alone, and combined oxygen and DPA treatment resulted in an activity significantly lower than that observed after hyperoxia alone (Figure 5).

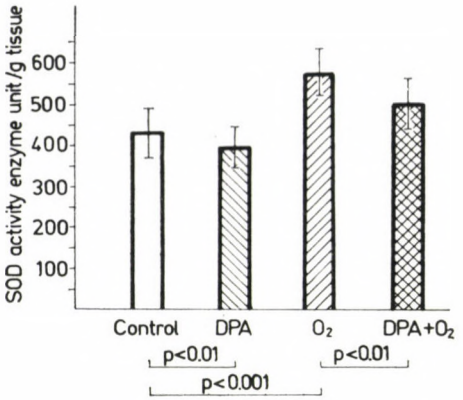

FIG. .4 Superoxide dismutase activity (SOD) in the lung of newborn rats after D-penicillamine (DPA), oxygen (O_2) and combined DPA + O_2 treatment

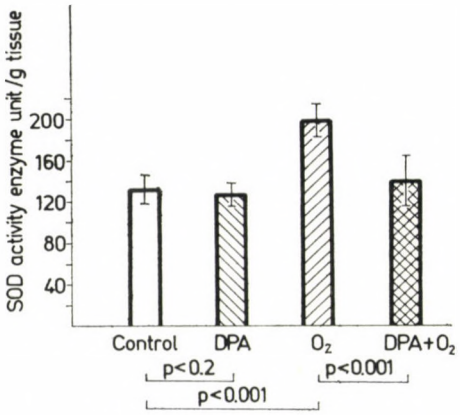

FIG. 5. Superoxide dismutase (SOD) activity in the retina and choroid of newborn rats after D-penicillamine (DPA), oxygen (O_2) and combined DPA + O_2 treatment

TABLE I

Superoxide dismutase activity in organs of the DPA treated newborn rat under the effect of oxygen

Tissue	SOD activity+, enzyme unit/g tissue			
	Control(n)	DPA(n)	O_2(n)	DPA+O_2(n)
Lung	436±60 (23)	394±53 (21)	571±63 (27)	498±74 (28)
Retina-choroid	132±15 (8)	126±11 (7)	188±19 (9)	140±25 (9)

n = number of experiments
+ = mean, ± = SD

Table I summarizes the SOD values for the lung and retina-choroidal tissues in the four experimental groups.

Discussion

Most authors investigating the cause of RLF and bronchopulmonary dysplasia agree in that hyperoxia and the consequent release of free oxygen radicals are of particular importance in their genesis. Kistler et al [19] studied the reaction of alveolar macrophages and polymorphonuclear cells to a prolonged inhalation of 85% oxygen. They found that oxygen consumption was significantly increased in both cell types after latex-phagocytosis. The extent of superoxide anion production was, however, significantly lower in the alveolar macrophages, which suggested a different reactivity against hyperoxia or a more rapid kinetics of their inactivation system [8, 9]. In recent years several reports have dealt with the changes of lung tissue following prolonged high concentration oxygen treatment [6, 14, 25, 26]. Smith et al [32] reported that young rats were relatively resistant against hyperoxia, and other studies [16, 17, 19] revealed that hyperoxia causes a significant proliferation in the alveolar wall of type II cells. In monkeys hyperoxia was observed to damage the type I cells and the majority of these damaged cells is replaced by type II cells. The second characteristic feature of hyperoxia was the development of pulmonary oedema on the 2nd — 3rd day of treatment. After a 12 day long exposure to oxygen at high concentration the extent of pulmonary oedema increased and the number of type II cells and mortality rate increased as well. In addition, it was shown that prolonged hyperoxia enhanced proteolysis in the lung, causing thus a reduction of enzyme synthesis [5, 11, 33].

Hyperoxia activates several protective mechanisms in the lung. Oxygen at high concentration increases the amount of oxidizable substrates, and the activity of biological systems playing a role in the inactivation of free oxygen radicals also increases [4]. According to Scholz and Rhoades [33], the following antioxidant systems are present in the lung: NADH--system and the related dehydrogenase enzymes glucose-6-phosphate-dehydrogenase, SOD, glutathione peroxidase and glutathione reductase.

The ability of newborn animals to protect themselves against oxygen toxicity is based on the rapid increase of antioxidant capacity [4]. The most striking increase of SOD activity occurs in the first 24 hours of oxygen breathing, whereas later only a slight increase in enzyme activity occurs [1].

The present results showed that (i) SOD activity increased in all experimental animals after protracted hyperoxia. Thus, oxygen at high concentration exerts an intensive inductive effect due probably to the release of free oxygen radicals; (ii) DPA was found to reduce the SOD production induced by hyperoxia.

Several explanations can be found for the latter finding. The red-violet copper-DPA complex has been shown to have SOD activity [24]. While DPA alone cannot inactivate the free oxygen radicals, the red-violet complex may be effective in the inactivation process, similarly as with the metallo-superoxide dismutases. Assuming that such a complex is formed in the animals after DPA treatment, during hyperoxia the tissues might use the complex for the inactivation of free oxygen radicals instead of their own protective enzymes. In other words, the copper—DPA complex may partially replace SOD in the biological protective process, which may intensify the protection against the toxic free radicals.

Recently it has been reported that DPA treatment increased the intracellular glutathione content in patients suffering from rheumatoid arthritis [28]. Glutathione is well known to have an important role in the stabilization of lysosomal and other membrane systems and in the inactivation of superoxide and other free radicals. This fact suggests that the DPA treated organism does not need an increase of own SOD activity. Most of the DPA administered *in vivo* produces mixed disulfide bonds with the disulfide containing serum proteins. In this way DPA might become a potent agent for the reduction of toxic free radicals and as a hydrogen-donor it may exert an important protective action against oxygen toxicity [29].

In conclusion, the present experiments suggest that DPA treatment, most probably as a result of complex biological processes, may play a role in the protection against oxygen toxicity by preventing the increase of SOD production.

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