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D-Penicillamine decreases the H_2O_2 and phenylhydrazine induced lipid peroxidation in the erythrocyte membrane

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The protecting effect of D-penicillamine against hydrogen peroxide and phenylhydrazine induced haemolysis and lipid peroxidation is discussed. It might represent a possible way of action of the drug in some neonatal disorders like hyperbilirubinaemia and retrolental fibroplasia.

In previous studies we have reported that D-penicillamine (DPA) had a significant therapeutic effect in infants with neonatal jaundice [2]. The overproduction of bilirubin is the consequence of haemolysis of different origin in the neonatal period. Lipid peroxidation has been suggested to be a mechanism of membrane damage in a number of red cell disorders leading to haemolysis. Since the susceptibility of red cell lipids to autoxidation is about three times higher in the newborn than in the adult [7], it was of interest to investigate the influence of DPA on lipid peroxidation of red cell membranes.

Goldstein et al. [1] developed a spectrofluorescent assay suitable for demonstrating red cell lipid peroxidation in vivo. In the present work we studied the inhibitory effect of DPA on the fluorescence of lipid containing extracts of red cells peroxidized in vitro by hydrogen peroxide, and in vivo by phenylhydrazine.

Materials and Methods

Chemicals were purchased from Reanal (Budapest, Hungary). All reagents were of analytical reagent grade. DPA (Metalcaptase®) was a gift from Knoll AG (Ludwigshaven, FRG).

Sensitivity of red blood cells to hydrogen peroxide was assessed according to the method of Mengel et al [4].

Lipid peroxidation of red blood cell membranes caused by H_2 in vitro was determined by the fluorescence assay described by Goldstein et al [1]. The same method served for the determination of phenylhydrazine-induced lipid peroxidation of red cell membranes in vivo.

Spectrofluorimetry was performed with a Hitachi—Perkin Elmer MPF 4 spectrofluorometer in the direct mode at 25°C with excitation and emission slits at 5 nm and sensitivity set at 30, an excitation maximum of 360 nm and an emission maximum of 440 nm. For lipid extraction a scaled-down version of the Rose and Oklander chloroform : isopropanol procedure was performed [6].

In vitro experiments

Blood was taken in heparinized syringes from healthy infants. Erythrocytes were washed three times in physiologic saline and made up in 5.0% solution. H_2O_2

haemolysis test was carried out under different conditions as follows.

1. Red blood cell suspension without preincubation with DPA;

2. Red blood cell suspension preincubated with DPA for 10 min;

3. Red blood cell suspension preincubated with DPA for 60 min. The final concentration of DPA was 0.1 mmol/l.

Triplicate samples of 0.5 ml cell suspension were mixed with equal volumes of 5% H_2O_2 in buffer at pH 7.4, incubated at 37°C for 15 minutes and than at room temperature for 2 hours and 45 minutes. Buffer blanks were carried throughout. 100% haemolysis was caused by distilled water. After centrifugation the supernatant was read at 540 nm after conversion of haemoglobin to cyanmethaemoglobin. Per cent haemolysis was calculated as:

$$\frac{E_{samlpe} - E_{blank}}{E_{100\%\ haemolysis} - E_{blank}} \times 100$$

Results of triplicate determinations were averaged.

Animal studies

Female CFY rats weighing 100—140 g were used. The animals were divided into three groups, each of which contained four to five animals.

On the first 3 days the animals in Group I were treated with 200 mg/kg DPA intraperitoneally (i.p.) daily while the animals in Groups II and III were injected with physiologic saline of the same volume.

On the following 6 days 200 mg/kgbw of DPA was administered daily to Groups I and II. The animals in Group III received physiologic saline i.p. instead of DPA. 30 minutes after each injection 20 mg/kg phenylhydrazine was given i.p.

Blood was collected by heart puncture, in heparinized syringes while the animals were under ether anaesthesia. Fluorescence assay was carried out before the start (Group 0) and at the termination (Groups I—III) of drug administration. The assay was routinely done in triplicate.

Results and Discussion

Table I shows the effect of DPA on haemolysis and peroxidation induced by H_2O_2 in a red blood cell suspension. Preincubation with DPA resulted in a significant decrease of both the haemolysis and the fluorescence of chloroform: isopropanol red cell lipid extracts induced by H_2O_2. The fluorescence activity and per cent haemolysis were significantly lower in the erythrocyte suspensions preincubated with DPA. The inhibitory effect depended on the incubation time.

Figure 1 shows the influence of DPA on fluorescence of red cell lipid extracts in rats injected with phenylhydrazine. In Groups II and III the fluorescence activities were higher at the termination of drug administration, than before the treatment. In Group I DPA pretreatment for three days resulted in a decrease of fluorescence, i.e. DPA prevented the phenylhydrazine induced lipid peroxidation. The inhibitory effect was significant statistically.

The mechanism of action of DPA in neonatal hyperbilirubinaemia is complex. DPA exerts an influence on heme metabolism, regulating the activity of heme-oxygenase which is the initial and rate-limiting enzyme in heme degradation. DPA treatment diminishes heme oxygenase activity in the liver of newborn animals, leading to a decrease of bilirubin production [5].

The present study showed that DPA prevents the H_2O_2 and phenyl-

TABLE I

Haemolysis and lipid peroxidation in erythrocyte membrane induced by hydrogen peroxide

Preincubation with D-penicillamine	% erythrocytes haemolyzed	Fluorescence units
None	39.98±3.53	89.00±6.68
10 min	16.17±1.35[a]	56.17±9.11[a]
60 min	11.95±1.32[a]	36.33±7.23[a]

[a] $p < 0.01$ (Student's t-test)

hydrazine induced lipid peroxidation of the erythrocyte membrane. This results in a decrease of haemolysis. It might be another, a direct mode of action of DPA moderating the hyperbilirubinaemia in newborns. Neonatal disorders related to oxygen toxicity, such as haemolytic diseases, retrolental fibroplasia, bronchopulmonary dysplasia, hyaline membrane diseases, have a multicausal aetiology. Their common feature, however, is a free radical induced lipid peroxidation leading to tissue damage. Thus, the decrease of membrane lipid peroxidation under DPA treatment may give an explanation for the beneficial effect of the drug in the prevention of retrolental fibroplasia in very low birthweight infants [3].

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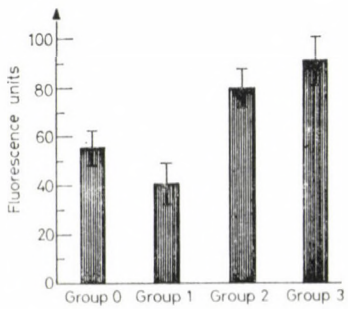

FIG. 1. Phenylhydrazine induced lipid peroxidation in erythrocyte membrane of D-penicillamine treated and control rats. (Details see in text)

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