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Effects of *In Vivo* Hyperoxia on Erythrocytes. III. *In Vivo* Peroxidation of Erythrocyte Lipid*

CHARLES E. MENGEL † AND HERBERT E. KANN, JR.

(From the Departments of Medicine of The Ohio State University, Columbus, Ohio, and Duke University, Durham, N. C.)

The use of oxygen under high pressure (OHP) for medical and surgical purposes (1–6) has stimulated renewed interest in, and provided a unique opportunity for study of, the biochemical and functional changes that occur during exposure of animals or humans to high oxygen environments. The possibility occurred to us that a hyperoxic environment might enhance oxidation of compounds in excess of that which would occur under normal physiologic conditions. A specific consideration was the possible *in vivo* peroxidation of unsaturated fatty acids.

Unsaturated fatty acids readily autoxidize *in vitro* to form lipid peroxides (7–10). The reaction takes place nonenzymatically in the presence of oxygen and ferrous ions. Studies in this laboratory and in others have linked *in vitro* peroxidation of erythrocyte lipid and hemolysis (11–18). Some observations had suggested that oxidation of unsaturated fatty acids might occur *in vivo* (11, 19, 20). However, to date the occurrence of lipid peroxidation *in vivo* has not been unequivocally demonstrated, and therefore its biologic significance has not been established.

Previous studies carried out in this laboratory (21–22) suggested that hemolysis occurring in mice exposed to OHP resulted from peroxidation of erythrocyte lipid. Evidence suggesting that a similar hemolytic mechanism could occur in humans was obtained from our findings in a patient who developed hemolytic anemia after a brief period of exposure to OHP (23). The studies reported herein demonstrate the *in vivo* formation of lipid peroxides in erythrocytes of mice exposed to oxygen under high pressure and implicate lipid peroxidation as a cause of hemolysis occurring under these conditions.

Methods

Mice. Male and female strain DBA/2 mice (6 to 9 months old, average weight 25 g) were used in all experiments. For each experiment 20 mice of comparable age, sex, and weight were exposed to hyperoxia. Ten of these were taken from a group of mice that had been maintained on a tocopherol-deficient test diet for a minimal period of 6 weeks. The other ten, which had been fed a standard chow preparation, were each injected intraperitoneally with 0.5 mg of alpha-tocopherol acetate 0.5, 3, or 18 hours before OHP exposure. In each experiment an equal number of control mice of comparable age, sex, and dietary status, but without exposure to hyperoxia, were studied. The weight of mice in each study group did not differ appreciably.

OHP procedure. Mice were placed in metal cages that had been coated with a saline glycerine solution (fire safety precaution) and which contained no food, water, or combustible material. The test cages were placed in a hyperbaric chamber. This chamber had a volume of 12 cubic feet and provided constant circulation of the gaseous environment, continual flushing by oxygen, and absorption of expired CO_2. Chamber pressure was brought to 60 pounds per square inch absolute pressure with 100% oxygen over a period of 5 to 10 minutes and was maintained for 1.5 hours. During this exposure none of the tocopherol-supplemented mice demonstrated central nervous system effects. Approximately 20% of chow-fed mice and 40% of tocopherol-deficient mice had seizures during these exposures. No mortality occurred in any group. No CO_2 could be demonstrated at several intervals tested with a micro-Scholander gas analyzer. Slow stepwise decompression was carried out over 20 minutes. There were no evidences, nor have there ever been in any of our studies, of untoward effects of pressure changes per se when the schedule cited here was used. Within 1 hour after removal from the cham-

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† John and Mary Markle Scholar in Academic Medicine; Director, Division of Hematology and Oncology, The Ohio State University, Columbus, Ohio.

Address requests for reprints to Dr. Charles E. Mengel, University Hospital, 410 West 10th Ave., Columbus, Ohio 43210.

ber, each mouse was exsanguinated by cutting exposed axillary blood vessels. This method (one of several tested) enabled us to obtain the greatest volume of blood (average, 1 ml) per mouse. Blood was collected in heparinized pipettes and immediately cooled to 4° C.

Routine and special hematologic studies. Microhematocrits, reticulocyte counts, and Heinz body preparations were performed on individual and pooled blood samples (24). Plasma was examined for evidence of gross hemoglobinemia. Whole blood methemoglobin levels were determined spectrophotometrically on individual and pooled blood samples (25). Glutathione content of pooled blood samples was quantitated with the method described by Beutler, Duron, and Kelly (26). Erythrocyte glucose 6-phosphate dehydrogenase activity was determined on pooled blood samples as described by Zinkham, Lenhard, and Childs (27), except that a change of absorbance of 0.001 was taken as 1 enzyme U, and activities were expressed as enzyme units per microliter cells per minute. Erythrocyte acetylcholinesterase activity was determined on pooled blood samples by using a method described by Ellman, Courtney, Andres, and Featherstone (28). A change of absorbance of 0.001 was taken as 1 enzyme U, and values were expressed as enzyme units per microliter cells per minute. Erythrocyte catalase activity was determined on pooled blood samples as described by Feinstein (29).

Hydrogen peroxide hemolysis test. Details of the method used for determining *in vitro* lytic sensitivity of erythrocytes to hydrogen peroxide have been described previously (22). All glassware used was washed with concentrated nitric acid and hydrogen peroxide (1,000 ml acid plus 5 ml 30% H_2O_2) and thoroughly rinsed with thrice-deionized water. Hydrogen peroxide solutions, 0.01% to 0.1% in a $KH_2PO_4 = NaOH$ buffer (pH 7.4), were stored away from light at 4° C. Each H_2O_2 solution was titrated iodometrically for actual peroxide content before use and readjusted to the desired concentration if necessary. Suspensions of erythrocytes in physiologic saline were then incubated with varying concentrations of H_2O_2 (0.01% to 0.1%) at 37° C for 15 minutes and at room temperature for 2 hours and 45 minutes. At completion of incubation, per cent hemolysis was determined spectrophotometrically on triplicate samples at each H_2O_2 concentration.

Lipid peroxide determinations. Lipid peroxides in erythrocytes were determined by measuring the pink chromogen (absorbance maximum, 535 mμ) formed by the reaction of 2-thiobarbituric acid (TBA) with a breakdown product of lipid peroxides, malonylaldehyde (30, 31). Erythrocytes from mice in each study group were washed twice in physiologic saline. Then, 0.16- or 0.2-ml portions of washed erythrocytes were mixed well with 1.5 ml 10% trichloroacetic acid. The mixture was filtered through Whatman no. 1 paper. Thiobarbituric acid (0.67% in water) was added to portions of the filtrate (usually 0.6 or 0.8 ml) in a ratio of 1.2 to 1. The mixture was heated in a boiling water bath for 15 minutes, then cooled to room temperature. Absorption spectra were taken and the absorbance at 535 mμ recorded.

Lipid peroxide levels in plasma were determined by combining 1 ml of plasma with 1 ml of 10% trichloroacetic acid and filtering the mixture through Whatman no. 1 paper. One ml of the filtrate was mixed with 1.2 ml of the thiobarbituric acid solution, and lipid peroxides were determined as outlined in the preceding paragraph.

Since it has not been possible to prepare a standard solution of unsaturated fatty acid peroxides, a standard absorption curve for malonylaldehyde was prepared using 1,1,3,3-tetraethoxypropane (TEP), a compound that hydrolyzes to 1 mole of malonylaldehyde and 4 moles of ethanol (32). With this curve as a standard an absorbance of 0.1 in the TBA reaction was calculated to be equivalent to 8 mμmoles of malonylaldehyde. Although many other aldehydes and ketones give some color with the TBA reagent, they fade rapidly and have different absorption maximums or low extinction coefficients (33).

Saturated fatty acids do not peroxidize. Malonylaldehyde is derived primarily from those unsaturated fatty acids which contain three or four unsaturated bonds, such as arachidonate and linolenate (34). Since these represent only a fraction of the total unsaturated fatty acids in naturally occurring lipids, this method measures only a portion of the total peroxidized unsaturated fatty acids. For example, Hochstein and Ernster found that malonylaldehyde levels accounted for only approximately 5% of the total oxygen consumed during peroxidation of lipids in rat liver microsomes (35). Most investigators agree, however, that this method may be used as a measure of lipid peroxidation (36–39).

Chromatographic studies. The chromatographic characteristics of the 2-thiobarbituric acid chromogen formed in erythrocytes of tocopherol-deficient mice exposed to OHP were compared to chromogens formed by reacting 2-thiobarbituric acid with acid-hydrolyzed TEP or with several types of lipid (arachidonic acid, lipid extracted from human erythrocytes, cod liver oil) that had been exposed to ultraviolet radiation. [Ultraviolet radiation causes peroxidation of lipids (38, 39).] Fifty μl of the clear pink pigment formed in each case after reaction with TBA was applied to Whatman no. 1 filter paper, chromatographic grade, 20 × 15 cm. The upper layer of a phenol-isopropanol-formic acid-water (80:10:10:100 parts by volume) mixture was used as a solvent after standing at room temperature for 24 hours (31, 40), and the chromatograms were developed for 90 minutes in an ascending system. After drying at room temperature, chromatograms were initially examined for visible pigment spots and fluorescent material. The pink pigments were eluted from the paper in small portions of 10% trichloroacetic acid in a boiling water bath. After cooling to room temperature, absorbance spectra were obtained.

Materials included tocopherol-deficient test diet,[1] alpha-tocopherol acetate,[2] 5,5′-dithiobis-(2-nitrobenzoic acid),[3]

[1] General Biochemicals, Chagrin Falls, Ohio.

[2] U. S. Vitamin and Pharmaceutical Corp., Arlington-Funk Laboratory, New York, N. Y.

[3] Aldrich Chemical Co., Milwaukee, Wis.

TABLE I

*Hematologic values and lipid peroxide levels in erythrocytes from mice**

Study group†	Hematocrit	Per cent reticulocytes	Appearance of plasma	Lipid peroxides‡
	%			
Tocopherol-deficient (14)	45–50	0.2–1.5	Normal	0
Chow-fed, tocopherol-supplemented (14)	44–51	0.2–1.6	Normal	0
Tocopherol-deficient +OHP (14)	14–24	9–16	Bright red	36–50
Chow-fed, tocopherol-supplemented +OHP (14)	47–50	0.2–1.4	Normal	0
Tocopherol-deficient, tocopherol-supplemented +OHP (5)	46–49	0.1–1.2	Normal	0

* Values were obtained from pooled blood samples of ten mice in each experimental group.
† The number after each group indicates the number of experiments; +OHP designates those mice exposed to oxygen under high pressure.
‡ Millimicromoles malonylaldehide per milliliter erythrocytes.

acetylthiocholine,[4] hydrogen peroxide,[5] 2-thiobarbituric acid,[6] and tetraethoxypropane.[7]

Results

The effect of *in vivo* OHP on mouse red cells is shown in Table I. Before OHP exposure no significant differences of hematologic indexes were noted between tocopherol-deficient and tocopherol-supplemented mice. Mice that had been maintained on the tocopherol-deficient diet for 4 months had normal hematocrits and showed no evidence of hemolysis before OHP exposure. During OHP, hemolysis (fall of hematocrit and marked hemoglobinemia) occurred in tocopherol-deficient mice. Whereas hematocrit values varied among individual mice, each tocopherol-deficient mouse exposed to OHP showed clear-cut evidence of hemolytic anemia. No evidence of hemolysis during OHP was noted in mice supplemented with tocopherol, 0.5, 3, or 18 hours before exposure to OHP. Neither sex nor age (in the range studied, 6 to 10 months) affected lytic sensitivity to OHP. When blood of individual mice was studied, no correlation was noted between central nervous system manifestations and severity of the hemolysis in the tocopherol-deficient group.

Red cells in Wright's-stained blood films showed moderate size and shape variations with some fragmentation of cells only in tocopherol-deficient mice exposed to OHP. No significant numbers of spherocytes were seen in any of the blood films.

Lipid peroxides were present in erythrocytes obtained from tocopherol-deficient mice immediately after exposure to OHP. None were found in erythrocytes from tocopherol-deficient mice not exposed to OHP. No lipid peroxides were detected in erythrocytes of tocopherol-supplemented mice either before or after OHP. Plasma of tocopherol-deficient mice exposed to OHP contained only trace amounts of lipid peroxides.

Since no red cell, tissue, or plasma tocopherol assays were performed, it was necessary to establish that the differences observed in the mice resulted from variations in tocopherol status per se rather than from dietary variation in some other unknown factor. As illustrated by the fifth study group in Table I, administration of 0.5 mg of alpha-tocopherol acetate to tocopherol-deficient mice 1 hour before OHP completely prevented the effects of OHP in red blood cells (RBC). From these data it seemed that tocopherol, its metabolites, or some unidentified component of the preparation accounted for the differences observed when mice were exposed to OHP.

Methemoglobin content of blood was normal in tocopherol-deficient and tocopherol-supplemented mice before OHP (less than 0.2%) and was not increased after OHP. No Heinz bodies were present in RBC from either group of mice before or after OHP exposure.

[4] Nutritional Biochemicals, Cleveland, Ohio.
[5] Becco, Buffalo, N. Y.
[6] Eastman Organic Chemicals, Rochester, N. Y.
[7] Kay-Fries Chemicals, Inc., New York, N. Y.

TABLE II

*Erythrocyte glucose 6-PO_4 dehydrogenase (G-6-PD) activity and reduced glutathione content in each mouse study group**

Study group	G-6-PD		GSH	
	Range	Mean ± SD	Range	Mean ± SD
	enzyme U/μl cells/min		*mg/100 ml erythrocytes*	
Tocopherol-supplemented	9.5–11.0	10.3 ± 0.6	47–54	49.5 ± 3.7
Tocopherol-deficient	10.2–12.0	10.8 ± 1.5	46–55	50 ± 3.7
Tocopherol-supplemented +OHP	10 –11.8	10.8 ± 0.7	48–51	49.3 ± 1.2
Tocopherol-deficient +OHP	12 –16	14.3 ± 1.7†	62–70	66.5 ± 3.4‡

* These values were obtained from pooled blood samples of ten mice in each experimental group. Figures represent results obtained in five separate experiments.
† $p < 0.025$.
‡ $p < 0.005$.

As shown in Table II, reduced glutathione content of blood and glucose 6-PO_4 dehydrogenase (G-6-PD) activity of erythrocytes were consistently and significantly elevated in tocopherol-deficient mice exposed to OHP.

No differences in erythrocyte catalase activity were noted between tocopherol-deficient and tocopherol-supplemented mice before exposure to OHP. No change of catalase activity occurred in either group during exposure to OHP.

Acetylcholinesterase activity of mouse erythrocytes was not affected by tocopherol deficiency or tocopherol supplementation. No significant changes in enzyme activity occurred in erythrocytes of tocopherol-supplemented mice exposed to OHP. An increase in acetylcholinesterase activity observed in red cells of tocopherol-deficient mice exposed to OHP correlated with the degree of reticulocytosis after OHP (12 to 24 EU per μl cells per minute before hyperoxia and 32 to 38 EU per μl cells per minute after hyperoxia).

The *in vitro* lytic sensitivity of erythrocytes to hydrogen peroxide (in each study group) is shown in Figure 1. Increasing concentrations of H_2O_2 produced progressively greater hemolysis. Erythrocytes from tocopherol-deficient mice were very sensitive to the lytic effect of hydrogen peroxide, whereas erythrocytes from tocopherol-supplemented mice showed minimal hemolysis at all concentrations of H_2O_2. Those erythrocytes from tocopherol-deficient mice exposed to OHP that did not lyse during OHP were consistently more resistant to the *in vitro* hemolytic effect of H_2O_2 than erythrocytes from tocopherol-deficient mice not exposed to OHP.

Further studies were carried out to determine whether the lipid peroxides found in erythrocytes of tocopherol-deficient mice exposed to OHP had been formed *in vivo* during exposure of the mice to OHP, or *in vitro* as the erythrocytes were manipulated in the presence of atmospheric oxygen. Erythrocytes of tocopherol-deficient mice formed large quantities of lipid peroxides *in vitro* and were lysed when exposed to *1*) bubbled oxygen at 37° for 6 to 12 hours, *2*) 100% oxygen at 60 pounds per square inch absolute pressure at 37° C for 1 and 12 hours, *3*) 0.1% hydrogen peroxide at 37° C for 3 hours, or *4*) ultraviolet radiation [42 cm below two Westinghouse Sterilamps G1 T8 in round bottom quartz flasks for 6 hours at 25° C (Table III)].

To determine the effect of prior *in vivo* tocopherol, deficient mice were each given 0.5 mg of alpha-tocopherol acetate intraperitoneally 1 hour

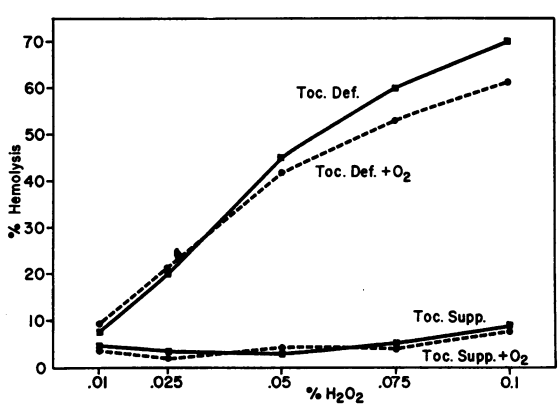

FIG. 1. IN VITRO LYSIS OF MOUSE ERYTHROCYTES BY HYDROGEN PEROXIDE. $+O_2$ indicates those erythrocytes taken from mice after exposure to oxygen under high pressure. Each point represents the average value of several experiments using pooled blood samples of several mice in each. Toc. def. and toc. supp. = tocopherol-deficient and tocopherol-supplemented mice, respectively.

TABLE III

*Effect of in vitro oxidant stresses on red cells of mice**

Oxidant stress	Tocopherol-deficient mice†		Tocopherol-deficient mice supplemented with alpha-tocopherol‡	
		% lysis		% lysis
Control cells in saline§	0	0	0	0
Bubbled oxygen	84	25	0	2
Oxygen under high pressure (12 hours)	80	12	0	2
Oxygen under high pressure (1 hour)	20	5	0	0
H_2O_2	120	70	0	0
Ultraviolet light	98	54	0	0

* Values given were obtained from pooled blood of ten mice of each study group in a single experiment. All determinations were carried out in triplicate.
† First column indicates millimicromoles malonylaldehyde per milliliter erythrocytes.
‡ First column indicates millimicromoles malonylaldehyde per milliliter cells.
§ Appropriate for each special incubation procedure.

before bleeding. Blood was collected in pipettes that had been rinsed with physiologic saline containing alpha-tocopherol emulsified in Tween-80 and physiologic saline (0.5 mg per ml), and all subsequent steps of the TBA test were performed with solutions containing alpha-tocopherol (0.5 mg per ml). When these red cells were subjected to the oxidant stresses listed above, each of which is capable of peroxidizing lipid, no significant lysis or lipid peroxidation occurred.

Since tocopherol as we used it had prevented *in vitro* lipid peroxidation by these agents, we reasoned that it should also prevent any *in vitro* peroxidation of lipid by atmospheric oxygen in erythrocytes of mice exposed to OHP. Accordingly, tocopherol-deficient mice were exposed to OHP in the routine manner and given 0.5 mg ip of alpha-tocopherol immediately after decompression. One hour later blood was collected in tocopherol-rinsed pipettes, and lipid peroxides were determined by using solutions containing alpha-tocopherol and saline mixtures as described above. As shown in Table IV, there were no differences in lipid peroxide levels between the tocopherol-deficient mice that were bled immediately after OHP and those which were given the tocopherol after OHP but before bleeding. No additional lipid peroxide formation occurred when erythrocytes of these animals were subsequently exposed to H_2O_2, oxygen, and ultraviolet radiation. Thus, the lipid peroxides found in erythrocytes of tocopherol-deficient mice exposed to OHP must have been formed *in vivo*.

TABLE IV

*Lipid peroxide levels in mouse erythrocytes**

Mouse study group	Lipid peroxides†
Tocopherol-deficient	0
Tocopherol-deficient +OHP	32–39
Tocopherol-deficient +OHP (given tocopherol before bleeding)	34–38
Tocopherol-deficient +OHP (given tocopherol before bleeding, blood collected and washed in tocopherol-saline mixture)	32–41
Tocopherol-supplemented (before and after OHP)	0

* Values were obtained on pooled blood of ten mice in each experimental group, and the range of three separate experiments is given.
† Millimicromoles malonylaldehyde per milliliter erythrocytes.

Results of chromatographic studies of the pink thiobarbituric acid pigment are shown in Figure 2. In the separation system used, the pink pigment formed by reacting 2-thiobarbituric acid with erythrocytes of tocopherol-deficient mice exposed to OHP was chromatographically identical to that formed when TBA was combined with malonylaldehyde or with other peroxidized lipids. For all pink pigments eluted from the paper, absorbance spectral peaks (i.e., maximal absorption at 535 mμ) were identical. Lipid from red cells of tocopherol-deficient mice not exposed to OHP formed no pink pigment with TBA and gave a

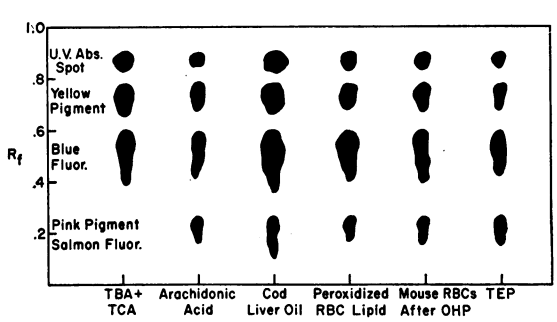

FIG. 2. SCHEMATIC DIAGRAM OF CHROMATOGRAPHIC BEHAVIOR OF PIGMENTS FORMED BY REACTIONS WITH 2-THIOBARBITURIC ACID. UVabs. = ultraviolet absorption; TBA + TCA (2-thiobarbituric acid + trichloroacetic acid) = blank; RBC = red blood cells; OHP = oxygen under high pressure, and TEP = 1,1,3,3-tetraethoxypropane.

chromatographic pattern identical to that of the blank (trichloroacetic acid + TBA).

Discussion

Although the clinical and histopathologic features of oxygen toxicity have been described in detail (41–52), the primary mechanism of cell damage by high oxygen tensions has not been elucidated. The development and use of oxygen under high pressure in hyperbaric chambers for medical purposes have made these effects of considerable practical importance since the *in vivo* levels of alveolar, venous, and arterial blood oxygen tensions reached during OHP far exceed those which are achieved by any other means (53–56). Aside from studies relating to erythropoiesis, the *in vivo* effect of increased oxygen tension on erythrocytes has received little attention. Its relevance to human clinical situations had not been considered until recently when several volunteers maintained in simulated space capsule environments (100% O_2 at low atmospheric pressures) developed evidences of red cell damage (increased osmotic fragility) and hemolysis (fall of hemoglobin and elevation of reticulocytes and indirect-reacting bilirubin) (57, 58), and one patient developed hemolytic anemia after a brief exposure to OHP (23). The latter patient's red cells were similar to those of tocopherol-deficient mice with regard to increased lytic sensitivity to H_2O_2, increased lipid peroxide formation by H_2O_2, and their *in vivo* sensitivity to hyperoxia. Whether his susceptibility reflected a tocopherol-deficient state or some alteration of fatty acid content in his erythrocytes was not decided. Many other studies were helpful only in ruling out various possibilities.

Work in other laboratories and our own studies had linked high oxygen tensions, erythrocyte lysis, and lipid peroxidation *in vitro* (11–17), and previous studies in this laboratory had suggested their relationship *in vivo* (21–23). The present studies establish the fact that peroxidation of erythrocyte lipid can occur *in vivo* during exposure to oxygen under high pressure. In these studies, *in vivo* erythrocyte lipid peroxidation has been invariably associated with hemolysis.

These effects were noted only in mice fed a tocopherol-deficient diet. Since no tocopherol assays were performed on erythrocytes, plasma, or other tissues of these mice, the question of their tocopherol status merits comment. Excellent evidence of their tocopherol deficiency was provided by the accentuated lysis and lipid peroxidation of their erythrocytes by hydrogen peroxide, as compared to normal mouse erythrocytes (11, 13, 14, 19). Whereas susceptibility to lipid peroxidation can be increased by diet-induced alterations in composition of erythrocyte lipid, specifically by an increase in erythrocyte unsaturated fatty acid content, this was unlikely in these experiments since the diet contained 10% lard rather than unsaturated fatty acids. The complete prevention of hemolytic anemia and erythrocyte lipid peroxidation during OHP by administration of tocopherol to the tocopherol-deficient mice shortly before their exposure to OHP made it unlikely that the differences between the two groups of mice reflected a diet-induced alteration of some nonlipid component of the erythrocyte. The dose of tocopherol used, however, is better considered "pharmacologic" rather than "physiologic," and the minimal dose required has not yet been established. In our previous studies, chow-fed mice (presumably of physiologic tocopherol status) did not develop overt hemolysis or peroxidation of RBC lipid during equivalent exposures to OHP.

Therefore, although probably an effect of tocopherol deficiency, the exact mechanism for susceptibility to *in vivo* lipid peroxidation has not been definitely established by these studies. Since this issue is of fundamental importance, we are presently initiating additional studies using more rigidly controlled dietary conditions, sequential detailed analyses of red cell lipid during the study period, and detailed tocopherol supplementation experiments to determine the minimal effective dose for prevention of lysis and lipid peroxidation *in vivo*.

A frequent criticism of earlier studies of lipid peroxidation has been the failure to consider the ability of atmospheric oxygen to peroxidize unsaturated fatty acids *in vitro*. Since determinations of lipid peroxide content always involved manipulations during which tissue was exposed to atmospheric oxygen, it always seemed possible that any lipid peroxides found could have been formed *in vitro*. Our observation that levels of erythrocyte lipid peroxides were not decreased when alpha-tocopherol was administered after

OHP, but before exsanguination (a maneuver we proved effective in preventing *in vitro* lipid peroxidation) established their formation *in vivo*. They were not formed as a result of hemolysis, since lipid peroxides were demonstrated in remaining intact erythrocytes.

That the pink pigment formed during incubation with TBA was in fact a product of peroxidized unsaturated lipid in the erythrocytes of mice exposed to OHP seemed likely from its chromatographic characteristics; the compound was chromatographically identical to that formed when other peroxidized lipid or malonylaldehyde was reacted with TBA.

No more than trace quantities of lipid peroxides were present in the plasma of mice at a time when their red cells contained relatively large amounts. In this regard, recent preliminary studies in this laboratory have shown that plasma not only inhibits peroxidation of lipid but also destroys formed lipid peroxides relatively rapidly (59).

None of the data from these experiments indicated that hyperoxic hemolysis occurred because of an overload of the hexose-monophosphate shunt. No methemoglobin or Heinz body formation occurred, and no decline in reduced glutathione content or G-6-PD activity was observed. The rise in reduced glutathione content and G-6-PD activity of blood from tocopherol-deficient mice exposed to OHP coincided with the reticulocytosis. The impressive rise in reticulocytes of tocopherol-deficient mice exposed to OHP occurred within 2 hours of the onset of hyperoxic exposure. The reticulocytes appeared characteristic and were identical by appearance to those observed after blood loss or induction of hemolysis by other means. They in no way resembled Heinz bodies or other types of inclusions. The speed of this response was more suggestive of a sudden shift of reticulocytes from the bone marrow into the circulation rather than an increase in marrow activity. Although this issue must remain unsettled at the moment, recent investigations in our laboratory have shown a marked increase of erythropoietic activity of the marrow in similarly exposed animals that has occurred simultaneously with reticulocytosis.

The exact relationship of lipid peroxidation to cell damage has not been defined. Two major possibilities exist. Hemolysis might occur as a direct consequence of peroxidation of unsaturated fatty acids in erythrocyte membranes. Peroxidation followed by rupture of double bonds in carbon chains could lead to anatomical defects in the cell membrane with subsequent alteration of permeability characteristics and eventual cell lysis. This mechanism has been postulated in lysis of mitochondria (60–61) and microsomes (33) under specific conditions. Alternatively, lysis could reflect a secondary effect of lipid peroxide formation, since the damaging effect of lipid peroxides on proteins, enzymes, and metabolic pathways has been well documented (62–64). With regard to this possibility, we have demonstrated changes in human erythrocyte glycolytic intermediates after prolonged *in vivo* exposure to OHP that suggested inhibition of the sulfhydryl-bearing enzyme glyceraldehyde 3-phosphate dehydrogenase (65). We have also observed decreased activity of erythrocyte acetylcholinesterase (presumed to be a sulfhydryl-bearing enzyme) in dogs exposed to OHP (66).

We suggest, therefore, that lipid peroxidation is the initial biochemical event induced by hyperoxia, and its direct effect and the toxic effects of lipid peroxides at other sites may combine to cause cell and tissue damage.

Summary

Studies of the effect of *in vivo* oxygen under high pressure on erythrocytes were carried out in tocopherol-deficient and tocopherol-supplemented mice. Hemolysis occurred only in tocopherol-deficient mice exposed to oxygen under high pressure. *In vitro* lytic sensitivity of red cells to hydrogen peroxide paralleled their *in vivo* lytic sensitivity to hyperoxia.

These studies established the *in vivo* formation of lipid peroxides in tocopherol-deficient mice during hyperoxia, an effect that preceded hemolysis. The relationship between hemolysis and lipid peroxidation was discussed.

Acknowledgments

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