Effect of hypervitaminosis A on hemolysis and lipid peroxidation in the rat

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Abstract Erythrocytes from rats fed large doses of Vitamin A alone, or large doses of vitamin A and vitamin E or diphenyl-*p*-phenylene diamine (DPPD) were studied for H_2O_2-induced hemolysis. The vitamin A-dosed rats were more susceptible than normal rats to H_2O_2-induced hemolysis. Hemolysis was not accompanied by lipid peroxidation. Nevertheless, the antioxidants vitamin E and DPPD inhibited hemolysis in erythrocytes from vitamin A-dosed rats. These antioxidants had the same inhibitory effect when they were included in the diet or added to erythrocyte suspensions in vitro. Erythrocytes from vitamin A-dosed rats with or without added vitamin E or DPPD were less susceptible than the erythrocytes from normal rats to osmotic challenge, showing that vitamin A was present in levels sufficient to alter the structure of the erythrocyte membrane. These studies show that oxidative hemolysis occurs when the erythrocyte membrane is modified. Furthermore, this oxidative hemolysis is unrelated to lipid peroxidation.

Supplementary key words hydrogen peroxide · antioxidants · α-tocopherol · osmotic fragility

The role of vitamin A in lipid peroxidation and hemolysis has been suspected since Moore and Sharman (1) showed that erythrocytes from rats fed an excess of vitamin A were hemolyzed by dialuric acid just like erythrocytes from vitamin E-deficient rats (2). These data are difficult to reconcile with other studies (3, 4) which found that retinol acted as a lipid antioxidant in vitro. In a previous communication (5), we found that retinol-induced hemolysis in normal erythrocytes was not accompanied by lipid peroxidation. In the present investigation, we have examined the role of vitamin A and the classical antioxidants, vitamin E and diphenyl-*p*-phenylene diamine (DPPD), in oxidative hemolysis induced by hydrogen peroxide.

METHODS

Albino rats, male or female (150 g), of the Medical College strain were used. They were maintained on a balanced diet ad lib supplied by Hind Lever Company, Bombay. The experimental rats were fed 100,000 I.U. of retinol in 0.5 ml of groundnut oil by stomach tube on alternate days for 5 days; the control rats were given the same amount of oil alone. Other experimental groups received groundnut oil containing 100 mg of vitamin E or 25 mg DPPD in addition to 100,000 I.U. of retinol. The animals in both groups were pair-fed. On the tenth day, the rats in both groups were bled by heart puncture under light ether anesthesia. Blood was drawn into a heparinized syringe; the cells were separated, washed twice with saline–phosphate buffer (pH 7.4), made up to the required volume in the same buffer–saline, and stored at 4°C in the refrigerator. Hemolysis studies were conducted as described by Krishnamurthy and Kartha (5) except that a 1:20 dilution of the cells was used with 2.5% hydrogen peroxide (in buffer–saline) as described by Horwitt et al. (6). The preincubation period of 2 hr was omitted and 5-ml portions of the cell suspensions in saline–phosphate buffer (pH 7.4) were mixed with 5 ml of 5% hydrogen peroxide in stoppered 50-ml Erlenmeyer flasks. The flasks were incubated at 37°C for various times with gentle shaking once every 15 min. Aliquots of 1 ml each were withdrawn at the required time intervals for the estimations of hemolysis and for lipid peroxidation. Lipid peroxidation of the incubated cells was determined as described by Krishnamurthy and Bieri (7) and the extinction at 535 nm of the color due to the thiobarbituric acid was expressed as the TBA index. For the in vitro studies, required quantities of α-tocopherol or DPPD were dissolved in 0.2 ml of ethanol and added to the Erlenmeyer flasks; the cell suspensions and hydrogen peroxide were then added. The flasks were shaken vigorously for 30 sec and incubated. The control samples were similarly treated, but with 0.2 ml of

Abbreviations: TBA, thiobarbituric acid; DPPD, *N,N'*-diphenyl-*p*-phenylene diamine.

TABLE 1. Effect of added α-tocopherol and DPPD on the H_2O_2-induced hemolysis of erythrocytes from hypervitaminotic A rats

Time of incubation (min)	0	90	120	270
		hemolysis, %		
Vitamin A cells + H_2O_2[a] + 0.2 ml ethanol	3 ± 0.2	30 ± 3	46 ± 6 (0.03)	53 ± 8 (0.03)
+ 500 μg Vitamin E[b]	5 ± 0.2	3 ± 0.1	3 ± 0.1	5 ± 0.2
+ 100 μg DPPD[b]	3 ± 0.1	3 ± 0.1	3 ± 0.1	3 ± 0.1
Control cells + H_2O_2[a] + 0.2 ml ethanol	1.5 ± 0.1	3 ± 0.2 (0.01)	6 ± 0.5 (0.02)	10 ± 0.5 (0.03)

[a] Erythrocytes from hypervitaminotic A or control rats in the presence of 2.5% H_2O_2 in buffered saline.
[b] In 0.2 ml of ethanol.
Results are means ± SD of six experiments. 100% hemolysis was with distilled water. The mean TBA index of lipid peroxidation was 0.00 except where indicated, in parentheses.

ethanol alone. To study the integrity of the erythrocytes obtained from rats fed various supplements, osmotic fragility studies were carried out with various concentrations of saline as described by Dacie and Lewis (8).

RESULTS AND DISCUSSION

Erythrocytes from rats with hypervitaminosis A or from control animals were studied for both hemolysis and lipid peroxidation. Erythrocytes from the vitamin A-fed rats showed increased hemolysis but little lipid peroxidation when they were incubated with H_2O_2 (**Table 1**). Hemolysis was prevented by the addition in vitro of either α-tocopherol or DPPD (Table 1). Both hemolysis and lipid peroxidation data of erythrocytes from hypervitaminotic A rats and from the groups whose diets were supplemented with vitamin E or DPPD are given in **Table 2**. Hemolysis induced by H_2O_2 in vitamin A-fed rats was also prevented when either vitamin E or DPPD was included in the diet. These studies showed that the dietary vitamin A enhanced H_2O_2-induced hemolysis in the absence of significant lipid peroxidation. Furthermore, the enhanced H_2O_2-induced hemolysis was prevented by antioxidants, α-tocopherol and DPPD, both in vitro and in vivo. It may be concluded therefore, that oxidative hemolysis induced by H_2O_2 does not involve lipid peroxidation.

The effect of vitamin A on the integrity of erythrocytes was also studied by osmotic fragility. Dietary vitamin A clearly altered the erythrocyte membrane, since the cells were more resistant to osmotic challenge (**Table 3**). This effect was also observed when either vitamin E or DPPD was included in the diet. These experiments therefore show that the lipid antioxidants and vitamin A alter the erythrocyte

TABLE 2. H_2O_2-induced hemolysis and lipid peroxidation of erythrocytes from hypervitaminotic A rats supplemented with vitamin E or DPPD

Time of incubation, min	0	60	120	180	240
			hemolysis, %[a]		
Vitamin A cells + H_2O_2	5 ± 0.5 (0.02)[c]	18 ± 0.5 (0.04)	37 ± 0.5 (0.07)	47 ± 0.45 (0.05)	50 ± 1.2 (0.04)
Vitamin A +E[b] cells + H_2O_2	5 ± 0.6 (0.01)	7 ± 1.1 (0.01)	7 ± 1.0 (0.01)	8 ± 1.5 (0.01)	10 ± 1.8 (0.01)
Vitamin A + DPPD[b] cells + H_2O_2	5 ± 0.5 (0.02)	5 ± 0.5 (0.01)	7 ± 0.8 (0.02)	8 ± 0.6 (0.02)	12 ± 1.2 (0.02)
Control cells + H_2O_2	1.2 ± 0.2 (0.03)	3 ± 0.5 (0.03)	4 ± 1 (0.02)	6 ± 2 (0.03)	9 ± 3 (0.04)

[a] Results are means ± SD of 10 rats in each group.
[b] Erythrocytes from hypervitaminotic A rats supplemented with vitamin E or DPPD.
[c] Mean TBA index of lipid peroxidation is in parentheses.

TABLE 3. Hemolysis due to osmotic fragility of erythrocytes[a]

% NaCl	Dietary Group															
	Control				+ Vitamin A				+ Vitamins A and E				+ Vitamin A and DPPD			
	min				*min*				*min*				*min*			
	0	30	60	120	0	30	60	120	0	30	60	120	0	30	60	120
	hemolysis, %[b]															
1.0	7	10	15	20	3	4	7	10	6	4	5	10	2	5	7	15
0.9	6	7	7	13	2	4	7	8	3	5	6	10	2	4	6	9
0.8	6	10	11	15	3	5	6	8	2	4	5	8	2	6	10	13
0.7	7	11	11	16	3	6	6	8	3	4	5	14	3	4	6	16
0.6	10	13	14	17	3	7	7	9	4	6	10	16	3	7	12	22
0.5	18	22	24	27	12	13	14	16	12	15	18	22	8	9	10	16
0.45	48	51	57	57	26	30	31	35	35	40	43	59	12	14	15	45
0.4	85	87	87	87	80	78	83	80	80	86	86	84	44	48	49	81

[a] Cells, 0.25 ml, were suspended in 5 ml of saline solution (graded concentrations), incubated for the stated times, and the extent of hemolysis was determined. Distilled water produced 100% hemolysis.

[b] Results are means of five experiments (five rats per group in each experiment).

membrane and enhance the susceptibility of a non-lipid component of the membrane to oxidative stress. In the present study, lipid peroxidation has been ruled out as the causative agent in hydrogen peroxide-induced hemolysis of the vitamin A-rich erythrocytes. It is possible that hydrogen peroxide-induced hemolysis involves the oxidation of membrane sulfhydryl groups, as suggested by Mezick et al. (9). Antioxidants such as vitamin E or DPPD may then act by preventing such SH-group oxidation, thereby inhibiting the H_2O_2-induced hemolysis. The causal relationship between lipid peroxidation and oxidative hemolysis does not seem to be established, although in dietary vitamin E or selenium deficiency, such a relationship has been reported (2). In the present study it is shown that hemolysis can occur in the absence of lipid peroxidation.

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