A NEW FUNCTION FOR α-TOCOPHEROL IN ERYTHROCYTE METABOLISM

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ABSTRACT

Evidence for a new function of α-tocopherol in maintaining oxyhemoglobin in human RBC's is reported. In *in vitro* systems of human erythrocyte suspensions, α-tocopherol shifts the equilibrium to a steady-state level, in favour of oxy-hemoglobin, when incubated with cells containing different amounts of (12, 28, 56 or 100 % methemoglobin. This action is synergised in the presence of glucose, ascorbic acid and an $NADPH_2$ generating system.

INTRODUCTION

EDER *ET AL*[1] have reported that a steady-state concentration of about 1% methemoglobin is maintained in the red cell in normal humans. Among the various substances, in the plasma for methemoglobin reduction to oxyhemoglobin, glucose and lactate[2] and ascorbate[3] have been reported to be effective. Further, the enzyme NADH-cytochrome-b_5-oxidoreductase is also believed to have a role in methemoglobin reduction in the blood[4]. Ascorbic acid has been shown to function as an antioxidant in animals[5] and further the ascorbate is believed to have a role in methemoglobin reduction in adults. Since α-tocopherol functions metabolically chiefly as a liposoluble antioxidant, in this paper, we report the results of our studies on the effect of α-tocopherol, alone, and with ascorbic acid, glucose, and an $NADPH_2$ generating system on methemoglobin reduction and oxyhemoglobin oxidation in intact, human cells. Our results indicate that α-tocopherol, in physiological concentrations, show a definite role in reducing methemoglobin, to oxyhemoglobin, in *in vitro* systems of human RBC's, though, its effect is not as pronounced as shown by ascorbic acid and the α-tocopherol effect is synergised in the presence of ascorbate, glucose and an $NADPH_2$ generating system.

MATERIALS AND METHODS

Blood was collected from adult human volunteers, into 3.8% citrate solution, and the red cells were separated immediately, washed 2–3 times with saline phosphate buffer (pH 7.4) and stored in the same buffer at 4°C. Methemoglobin cells containing different amounts of methemoglobin (12, 28, 56 and 100%) were prepared by treating the packed cells with different concentrations of $NaNO_2$ (0.1 to 0.5%) in saline phosphate buffer as described by Taylor and Hochestien[8].

Hemoglobin conversion to methemoglobin and the reverse was studied essentially as described by Sullivan and Stern[3]. The red cells 5% were suspended in Krebs Ringer phosphate buffer (pH 7.4). The additives were prepared in the same buffer except α-tocopherol which was initially dissolved in 0.02 ml ethanol and mixed with buffer. The incubation mixture contained 2.5 ml of 5% suspension of cells in the buffer, 2 ml of buffer plus 1 ml of the buffered solution containing the additives or 1 ml of the buffer alone in the control samples. The effective concentration of packed cells in the system was 0.125 ml, in the total volume of 5.5 ml (32 mg oxyhemoglobin and 0.3 mg methemoglobin) initially. The reaction mixtures were incubated at 37°C in a water bath and at 0, 2.5 and 5 hourly intervals, 0.7 ml of the aliquots were withdrawn, analysed for oxyhemoglobin and methemoglobin following the procedure of Harley and Mauer[9] and Sullivan and Stern[3]. Oxyhemoglobin was measured by the increase in absorbance at 620 nm of the lysates following the addition of $Fe_3(CN)_6$ and methemoglobin by the decrease in absorbance at 620 nm after addition of CN^-.

RESULTS AND DISCUSSION

Table 1 gives the results of oxyhemoglobin methemoglobin equilibrium obtained after incubation of red cells with α-tocopherol (0.04 μM and 0.25 μM), L-ascorbate (10 mM) and glucose (5 mM). α-tocopherol in both the concentrations, at the end of 5 hr incubation produced 6% methemoglobin while with ascorbate 20% methemoglobin was formed. Both together lead to 13% methemoglobin formation. The presence of glucose in the above systems inhibited the formation of methemoglobin.

Table 1 *Oxyhemoglobin-methemoglobin equilibrium in red cells (100% oxyhemoglobin) incubated with α-tocopherol, ascorbate and glucose.*

Additions	Conc.	Percentage methemoglobin formed 2.5 hr	5 hr
None	—	1–2	1–2
α-tocopherol	0.04 μM	6	6
	0.25 μM	6	6
Ascorbate	10.00 mM	15	20
Glucose	5.00 mM	1–2	1–2
α-tocopherol + Ascorbate	0.04 μM + 10.00 mM +	10	13
α-tocopherol + glucose	0.04 μM + 5.0 mM	2	12
Ascorbate + glucose	10 mM + 5 mM	8	10
α-tocopherol + Ascorbate + glucose	0.04 μM + 10 mM + 5 mM	7	9

2.5 ml of 5% suspension + 2 ml buffer + 1 ml, buffer containing the additives; initially the cell suspension contained 100% methemoglobin; incubation at 37°C.

Table 2 gives the data obtained on the effect of the above three substances, on methemoglobin containing cells. Fresh red cells were treated with different amounts of $NaNO_2$ solution (0–0.5%) (in saline PO_4-buffer) and the methemoglobin cells harvested out, were incubated with the additives. Whatever be the initial methemoglobin concentration, at steady state equilibrium 20% and 75% oxyhemoglobin were reached at the end of 5 hr incubation, with α-tocopherol (0.04 μM) and ascorbate (10 mM) respectively; both together resulted in 80% oxyhemoglobin while the presence of glucose shifted the equilibrium still further, to the oxyhemoglobin formation. In a $NADPH_2$ generating system, consisting of NADP and glucose-6-PO_4, both α-tocopherol and ascorbate, shifted the equilibrium in favour of formation of more oxyhemoglobin, when incubated with cells containing 100% methemoglobin and glucose again synergised the above action. It may be mentioned that the methemoglobin containing cells, on incubation, even up to 5 hr at 37°C, without any additives did not change in its methemoglobin content (result not given in table 2).

The results of the present study confirm the reports of Sullivan and Stern[3] that an equilibrium of 75% oxyhemoglobin and 25% methemoglobin resulted in red cells when incubated with a concentration greater than physiological concentration of ascorbate, *i.e.* (10 mM) regardless of the oxyhemoglobin methemoglobin ratio at the beginning of the incubation. α-

Table 2 *Oxyhemoglobin methemoglobin equilibrium in methemoglobin containing cells, incubated with α-tocopherol, ascorbate, glucose and $NADPH_2$ generating system.*

Additions	% methemoglobin at zero time	Percentage of oxyhemoglobin formed 2.5 hr	5 hr
α-tocopherol	12	13	18
	28	13	20
	56	14	20
	100	17	20
Ascorbate	12	43	75
	28	44	73
	56	70	75
	100	75	75
α-tocopherol + glucose	12	18	25
	28	20	25
	56	20	25
	100	20	26
α-tocopherol + Ascorbate	12	47	80
	28	50	80
	56	75	80
	100	75	80
Ascorbate + glucose	12	48	85
	28	64	85
	56	86	86
	100	86	86
α-tocopherol + Ascorbate + glucose	12	51	95
	28	78	97
	56	85	97
	100	95	97
α-tocopherol + NADP + glucose-6-phosphate	100	25	35
Ascorbate + NADP + glucose-6-phosphate	100	40	83
α-tocopherol + glucose + NADP + glucose-6-phosphate	100	19	30
α-tocopherol + Ascorbate + NADP + glucose-6-phosphate	100	43	87
Ascorbate + glucose + NADP + glucose-6-phosphate	100	71	92
α-tocopherol + Ascorbate + glucose + (NADP + glucose-6-phosphate)	100	79	99

Red cells treated with $NaNO_2$ (0–0.5%) and resulting in 0, 12, 28 and 100% methemoglobin incubated with the additives α-tocopherol (0.04 μM) ascorbate (10 mM) glucose (5 mM). NADP-0, 0.04 mM, glucose-6-phosphate 10 mM were used. Results are a mean of 5 different experiments.

tocopherol is shown to have a similar property of inducing an equilibrium of 20% oxyhemoglobin and 80% methemoglobin when incubated with cells containing different amounts of methemoglobin initially. However with cells containing 100% oxyhemoglobin α-tocopherol induced only about 6% of methemoglobin formation. Glucose and $NADPH_2$ generating system in all the incubations, favoured formation of more HbO_2 than with α-tocopherol or ascorbate alone. It is significant that the effect of α-tocopherol is evident at a concentration of 0.04 μM which is of the order of α-tocopherol present in normal humans[10] while the effect of ascorbate has been shown to be negligible at the physiological concentration (0.1 mM).

The role of ascorbate at levels higher than physiological concentration in maintaining 75% oxyhemoglobin and 25% methemoglobin has been explained by the coupled oxidation of ascorbate and hemoglobin, by Spiegel *et al*[11] by the following 2 reactions:

$$AH^- + HbO_2 + H^+ \rightarrow A.^- + Met\,Hb + H_2O_2$$

$$AH^- + Met\,Hb \rightarrow A.^- + Hb + H^+$$

Ascorbic acid is impermeable to red cells while dehydroascorbate is capable of entering the cell[6] and therefore it was postulated that within the cells dehydroascorbate is converted to ascorbate through the action of the reduced glutathione which in turn is maintained by NADPH. The function of α-tocopherol in shifting the equilibrium from methemoglobin to oxyhemoglobin (table 2) may be through maintaining the ascorbate level by preventing its oxidation to dehydroascorbate within the cell. The finding that α-tocopherol synergises the effect of ascorbate in the above system confirms the above postulate. The synergestic effect of glucose may be explained as due to its stimulating effect on the hexose monophosphate shunt leading to higher rate of production of NADPH and a steady level of GSH. This is supported by the observation that the presence of NADPH in the system also synergised the effect of α-tocopherol and the simultaneous presence of glucose, ascorbate NADPH and α-tocopherol resulted in the conversion of 99% methemoglobin to oxyhemoglobin.

The results of our present study would therefore indicate that at the physiological level of α-tocopherol in blood it has a significant role for maintaining the oxyhemoglobin concentration with in the erythrocytes.

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ANNOUNCEMENT

AMRUT MODI RESEARCH AWARD FOR DR. DILBAGH RAI SRIDHAR

Dr Dilbagh Rai Sridhar, General Manager (R&D) IDPL Research Centre, Hyderabad has been selected for the Twelfth Annual Amrut Modi Research Award for the year 1981 for his outstanding contributions in the field of Pharmacy and Pharmaceutics. Dr Sridhar shares this award with Dr (Mrs.) M. R. Daichwal of C. U. Shah College of Pharmacy, Bombay. The award carries a cash prize of Rs. 10,000 and citation.