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EFFECT OF ASCORBATE ON RED BLOOD CELL LIPID PEROXIDATION

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The present study investigates the effect of ascorbate on red cell lipid peroxidation. At a concentration between 0.2 mmol – 20 mmol/1 ascorbic acid reduces hydrogen peroxide-induced red blood cell lipid peroxidation resulting in a marked decrease in ethane and pentane production as well as in haemolysis. Ascorbic acid also shows an antioxidant effect on chelated iron-catalyzed hydrogen peroxide-induced peroxidation of erythrocyte membranes. At a concentration of 10 mmol/1 ascorbic acid totally inhibits oxidative break-down of polyunsaturated fatty acids by radicals originating from hydrogen peroxide.

Our results indicate that ascorbate at the chosen concentration has an antioxidant effect on red blood cell lipid peroxidation.

Key words: ascorbic acid; lipid peroxidation; polyunsaturated fatty acids; alkane release

INTRODUCTION

Ascorbate interferes with peroxidative reactions acting either as prooxidant or antioxidant. Whereas the prooxidant capacity of ascorbate is generally explained by the reduction of ferric to ferrous iron, the mechanism of its antioxidant effect is still controversial.

Some people presume ascorbic acid to have a direct radical scavenging action[3,13] others suggest inhibition of propagation reactions by scavenging alkoxy radicals[16]. Performing the experiments designed here we tried to find out the cellular structures mainly protected by ascorbic acid and to get some information about the mechanism involved.

Methods

Red cells were isolated from the blood of healthy donors, as described by Beutler *et al.*[9], with microcrystalline cellulose and α-cellulose (1:1, w/w). Blood was drawn into

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one fifth volume of sodium citrate (stock solution 3.13%). Ghost membranes were isolated as described by Burton *et al.*[23]. Prior to the induction of peroxidation, red cell membranes were preincubated with ascorbic acid for 15 min. All experiments were performed with 10 mmol/1 hydrogen peroxide as peroxidizing agent. Erythrocyte suspensions (haematocrit: 2.5%) were incubated with 0.25 mmol/1 sodium azide for the inhibition of catalase. Lipid peroxidation of ghost membranes was initiated in the presence of 2.0 mmol/1 $FeSO_4$ and 4 mmol/1 EDTA[8]. Analysis of hydrocarbons in head space vials was performed as previously described[7]. Protein content of ghost membranes was estimated by the method of Lowry *et al.*[10]. Fatty acids in ghosts were analysed by using a column SP 2330 (Supelco) as described previously[22].

Results

Incubation of erythrocytes with ascorbic acid reduces the hydrogen peroxide induced release of alkanes (Table I).

The first decrease in gas production was found at a concentration of ascorbic acid of 0.2 mmol/1. With increasing concentration of ascorbic acid the alkane production further decreased. At a concentration of 10 mmol/1 no gas release could be measured in the head space vials (Table I).

Corresponding to the reduced ethane and pentane production, hydrogen peroxide-induced haemolysis decreased after incubation with ascorbic acid (Table II). Similar to the gas pattern the lowest concentration at which a reduction of haemolysis started was 0.2 mmol/1, maximal inhibition of haemolysis was found at 10 mmol/1.

To investigate possible gas production by a mixture of ascorbic acid with hydrogen peroxide the two substances were added to PBS pH 7.4 without showing any alkane release at all. After adding ferrous iron (2 mmol/1) and EDTA (4 mmol/1) to ascorbic acid (40 mmol/1) and H_2O_2 (80 mmol/1) a barely measurable amount of propane was found (data not shown). But under the conditions chosen in our experiments no gas release could be found on incubating ferrous iron (2 mmol/1) EDTA (4 mmol/1), ascorbic acid (20 mmol/1) and H_2O_2 (10 mmol/1). Therefore we looked for changes in gas production while incubating erythrocyte membranes with chelated iron ($FeSO_4$ 2 mmol/1 + EDTA 4 mmol/1) and hydrogen peroxide and ascorbic acid (Table III). In this reaction system as well, addition of ascorbic acid markedly reduced the hydrogen peroxide-induced alkane release.

TABLE I
Antioxidant effect of ascorbic acid

Ascorbic acid (mmol/1)	Ethane	Pentane
0	0.19 ± 0.3	0.79 ± 0.7
0.2	0.19 ± 0.04	0.64 ± 0.06
0.5	0.18 ± 0.04	0.63 ± 0.31
1.0	0.15 ± 0.03	0.38 ± 0.09
2.0	0.09 ± 0.04	0.30 ± 0.09
5.0	0.11 ± 0.05	0.25 ± 0.08
10.0	0.05 ± 0.04	0
20.0	0	0

Red cells (suspension in PBS pH 7.4 haematocrit 2.5%) were preincubated with ascorbate for 15 min. Thereafter, lipid peroxidation was induced by hydrogen peroxide (10 mmol/1) in the presence of azide (0.25 mmol/1) for 2 hours at 37°C. Alkanes expressed as nmol·gHb^{-1}·2 hrs^{-1}·Mean values ± SD.

n = 4.

TABLE II
Effect of ascorbate on hydrogen peroxide-induced haemolysis

Ascorbic acid (mmol/l)	Haemolysis in %	n
0	35.8 ± 7.5	38
0.2	28.8 ± 6.3	4
0.5	20.0 ± 5.4	4
1.0	11.0 ± 4.9	4
1.7	4.6 ± 1.6	4
2.0	2.8 ± 2.4	4
5.0	1.3 ± 1.5	4
10.0	0	4
20.0	0	4

Incubation procedure see Table I, mean values ± SD.

TABLE III
Effect of ascorbate on lipid peroxidation of red cell membranes

Addition	Ethane	Pentane	n
Fe^{++}-EDTA + H_2O_2 (10 mmol/l) + Ascorbate (20 mmol/l)	0.34 ± 0.13	0.71 ± 0.28	10
Fe^{++}-EDTA + H_2O_2 (10 mmol/l)	0.5 ± 0.12	2.45 ± 0.35	9

Red cell membrane suspensions were adjusted to a protein content of 0.08 mg/ml, 2 mmol/l $FeSO_4$, 4 mmol/l EDTA. 2 h incubation in PBS at 37°C, Alkanes expressed as nmol/10 mg membrane protein.

To further investigate the protective role ascorbic acid plays in reactions involving hydrogen peroxide-mediated peroxidation, we examined the fatty acid composition of erythrocyte membranes before and after incubation with hydrogen peroxide for 2 hours and the effect of the addition of ascorbic acid to the medium (Table IV). After incubation with H_2O_2 there was a marked drop in arachidonic acid (C 20:4) and docosahexaenic acid (C 22:6), therefore the relative proportion of saturated and monounsaturated fatty acids increased. On adding ascorbic acid (10 mmol/l) to the erythrocyte suspension no change in the fatty acid composition after incubation with H_2O_2 could be found.

DISCUSSION

Preincubation of red blood cells with ascorbic acid reduces the amount of hydrocarbon gases released when incubated with sodium azide and hydrogen peroxide. When looking at the changes in the gas pattern we first presumed vitamin C to interfere with scission reactions, i.e. altering the propagation process and changing the alkane release, but not actually protecting the red blood cells against peroxidation. The fact that H_2O_2-induced haemolysis decreased after preincubation with ascorbic acid, demonstrated the antioxidative role of vitamic C in our system.

Other parameters of peroxidative reactions like MDA and its precursors have also been found to decrease after the addition of ascorbic acid to the incubation medium[1,5,11].

TABLE IV
Effect of ascorbic acid on peroxidative break-down of red cell membrane fatty acids

Addition	C16:0	C18:0	C18:1	C18:2	C20:4	C22:6
Erythrocytes	23.3%	16.5%	16.8%	14.3%	21.5%	7.9%
Addition of H_2O_2 (10 mmol/1) and NaN_3 (0.25 mmol/1)	33.1%	15.9%	21.3%	19.6%	9.4%	0.8%
Addition of H_2O_2 (10 mmol/1) and NaN_3 (0.25 mmol/1) after incubation with ascorbic acid for 15 min	23.7%	15.7%	16.0%	13.3%	21.9%	9.4%

Fatty acids in red cell membranes expressed as % of all fatty acids shown.

In previous publications we have shown that ethane and pentane originate from the erythrocyte membrane during hydrogen-peroxide induced red blood cell lipid peroxidation[8,11]. Therefore, when observing this marked decrease in ethane and pentane production, we expected the effect of ascorbic acid to at least partially be the protection of the membrane against oxidative attack.

Incubating erythrocyte membranes with chelated iron and hydrogen peroxide, the alkanes released markedly decreased when ascorbic acid was added to the system.

With ascorbic acid at a concentration of 20 mmol/1 we were not able to measure any gas release in erythrocytes with hydrogen peroxide. In experiments using ghosts as substrates, of H_2O_2-induced peroxidative reactions, there is still some ethane and pentane production in spite of the same concentration of ascorbic acid being present in the system. These differences are presumably due to the intracellular enzymatic reduction capacity. In living cells there has to be an efficient system for vitamin C reduction because the vast majority of vitamin C is transported through the cellular membrane as dehydroascorbate and must be reduced using the hexosemonophosphate-shunt[2,12] for keeping up high NADPH and GSH levels to reduce dehydroascorbic acid[12]. The oxidised form of vitamin C can possibly be reduced in a kind of recycling process using the intracellular enzyme cascades. This hypothesis is supported by results presented by Trotta *et al.*[2] who found a 35% inhibition of peroxidation using extracellular ascorbate, whereas intracellular ascorbate and an activated hexose-monophosphate shunt resulted in a 86% inhibition.

In reaction systems using different substrates and different radical generating systems, ascorbic acid also showed an antioxidant effect, e.g. inhibition of phenylhydrazine or t — butylhydroperoxide[2] induced lipid peroxidation. Having localized the main protective effect of ascorbic acid on the membrane we looked for the structures in the membrane mainly affected by oxidative attack. A fatty acid analysis before and after H_2O_2 addition showed that only the highly unsaturated fatty acids were broken down during the peroxidative reactions. Addition of ascorbic acid to the system inhibited totally the oxidative break-down of polyunsaturated fatty acids.

There are several hypotheses to explain the antioxidant action of ascorbic acid. Some postulate a direct free radical scavenging capacity of vitamin C[3,13], others presume it to be an alkoxy-radical scavenger[16]. Some experiments[4,5,17,18,19,20] showed an

interaction between ascorbic acid and α-tocopherol, because vitamin C and vitamin E given together have a more effective antioxidant protection than the sum of both vitamins alone[5]. The presence of vitamin E in the erythrocyte membranes and the regeneration of it during peroxidative reactions by the addition of ascorbic acid could be the reason for the decreased gas release and polyunsaturated fatty acid break-down after H_2O_2 — induced radical formation.

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