J. Nutr. Sci. Vitaminol., **32**, 475–479, 1986

Free Radical Chain Oxidation and Hemolysis of Erythrocytes by Molecular Oxygen and Their Inhibition by Vitamin E

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(Received May 29, 1986)

Summary Erythrocytes of vitamin E-deficient rats and normal rats were oxidized at 37°C by molecular oxygen using a free radical initiator. The erythrocytes were oxidized by a free radical chain mechanism with kinetic chain length considerably larger than 1 and resulted in hemolysis. Vitamin E suppressed both oxidation and hemolysis, but the extent of hemolysis was determined primarily by the extent of oxidation independent of the presence or absence of vitamin E.

Key Words oxygen radicals, peroxidation, chain oxidation, hemolysis, vitamin E, antioxidant

It is now generally agreed that free radicals generated in biological systems play a toxicological role and, above all, toxicity by oxygen radicals has been suggested to be involved in a variety of biological events such as heart disease, cancer, and aging (*1*, *2*). Several kinds of active oxygen species are known, but one has to clearly distinguish between radical and non-radical species. For example, singlet oxygen is one of the active oxygen species, but it is not a radical and one singlet oxygen molecule reacts with one molecule of unsaturated fatty acid to yield one molecule of lipid hydroperoxide. On the other hand, one molecule of oxygen radicals can produce many lipid hydroperoxides by chain reactions. Therefore, whether free radical chain reaction proceeds and, if so, how long the kinetic chain length lasts are important questions. However, it has not been clearly established whether the biological molecules are oxidized by a free radical chain mechanism, partly because the rate of free radical generation has not been usually known and the kinetic chain length can never be calculated without knowing the rate of chain initiation. In this

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study, we have tried to answer the above questions in the peroxidation of erythrocyte membranes.

It has been observed that erythrocytes are susceptible to various oxidative stress (*3*, *4*), because erythrocyte membranes are composed of many polyunsaturated fatty acids and exposed to high concentration of oxygen and hemoglobin. It has been recently observed (*5*) that the erythrocyte ghost membranes are oxidized by molecular oxygen and that vitamin E suppressed this oxidation efficiently. We would like to report here that erythrocyte membranes are oxidized by a free radical chain mechanism which causes hemolysis and that vitamin E is effective as a chemical antioxidant in suppressing the peroxidation and hemolysis.

MATERIALS AND METHODS

Wistar strain male rats were fed on a vitamin E-deficient diet for 12 weeks. Blood was obtained from rats by cardiac puncture and collected in a heparinized tube. Erythrocytes were separated from plasma and buffy coat by centrifugation at $1{,}000 \times g$ for 10 min and washed three times with physiological saline. The concentrations of α-tocopherol in the erythrocyte membranes from vitamin E-deficient and control rats were measured as 25 and 308 μg/dl respectively.

2,2′-Azobis(2-amidinopropane) dihydrochloride (AAPH) was used as a water-soluble radical initiator to obtain a constant and measurable rate of chain initiation, which is essential for the quantitative kinetic study. The AAPH solution was prepared by dissolving an appropriate amount of AAPH in an aqueous solution containing 0.05 M NaCl and 0.01 M phosphate buffer (pH 7.3). Two ml of packed erythrocytes was suspended in 8 ml of an aqueous solution containing 0.125 M NaCl and 0.01 M phosphate buffer (pH 7.3). Then 10 ml of AAPH solution and 10 ml of erythrocyte suspension were mixed. An aliquot of the solution was taken into a 30 ml pyrex glass ampoule, connected to a pressure transducer (Model PMS-5M-2H, Toyoda Machine Works, Ltd., Japan) and the rate of oxygen uptake was followed under air at 37°C. The rest of the erythrocyte suspension was incubated under air in a water bath maintained at 37°C. An aliquot (0.5 ml) was taken out periodically to measure the extent of hemolysis spectrophotometrically as reported previously (*6*).

RESULTS AND DISCUSSION

The oxidation of the phospholipid bilayer by molecular oxygen initiated with AAPH proceeds by the following mechanism (*5*, *7*, *8*), where A is $HClHN=C(NH_2)\text{–}C(CH_3)_2$ and LH is polyunsaturated fatty acid moiety.

Initiation:

$$A\text{–}N=N\text{–}A \xrightarrow{k_d} [A\cdot N_2\cdot A] \longrightarrow (1-e)A\text{–}A \quad (1)$$

$$\searrow\ 2eA\cdot \quad (2)$$

$$A\cdot(\text{aqueous phase}) \xrightarrow{O_2,\ LH} AOOH + LOO\cdot(\text{in membrane}) \quad (3)$$

Propagation:

$$LOO\cdot + LH \longrightarrow LOOH + L\cdot \quad (4)$$

$$L\cdot + O_2 \longrightarrow LOO\cdot \quad (5)$$

Termination:

$$2\ LOO\cdot \longrightarrow \text{non-radical products} \quad (6)$$

The azo compound decomposes unimolecularly to give a nitrogen molecule and carbon-centered geminate radicals which are surrounded by a solvent cage. A fraction of the geminate radicals recombine in the solvent cage to give a dimer or disproportionation products (reaction 1), but the rest of them escape from the solvent cage (reaction 2), react with oxygen and attack the lipid to generate lipid radial, L·, in the membrane, which reacts rapidly with oxygen to give a lipid peroxy radical, $LO_2\cdot$ (reaction 3). The lipid peroxy radical attacks another lipid molecule to yield lipid hydroperoxide and simultaneously new lipid radical, which reacts with oxygen to give again the lipid peroxy radical (reactions 4 and 5). Thus, the reactions 4 and 5 continue repeatedly and the oxidation proceeds by a free radical chain mechanism and only one initiating radical can produce many lipid hydroperoxides and eventually causes membrane damage. The number of chain propagations per one initiating radical is called a kinetic chain length.

Figures 1 and 2 show the rates of oxygen uptake and hemolysis respectively during the oxidations of erythrocytes from vitamin E-deficient and control rats as 10% aqueous suspension at 37°C initiated with AAPH.

The rate of chain initiation is given by $2ek_d[\text{AAPH}]$, where e and k_d are the efficiency of free radical production from AAPH and the rate constant for unimolecular decomposition of AAPH respectively. The value of ek_d was assumed to be the same as that ($1.64\times10^{-7}\ s^{-1}$ at 37°C) for the erythrocyte ghost system

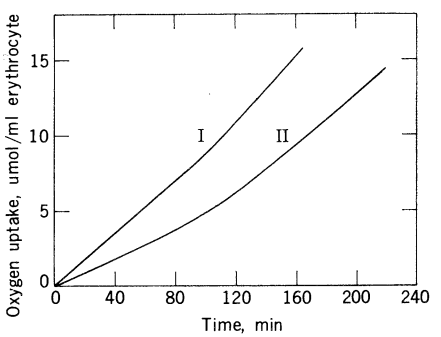

Fig. 1. Rate of oxygen uptake in the oxidations of erythrocytes of vitamin E- deficient rat (I) and control rat (II) as 10% suspension at 37°C initiated with 74 mM AAPH.

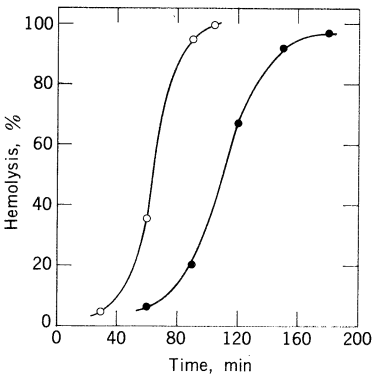

Fig. 2. Hemolysis during the oxidation of vitamin E-deficient rat (○) and control rat (●) erythrocytes as 10% suspension at 37°C initiated with 74 mM AAPH.

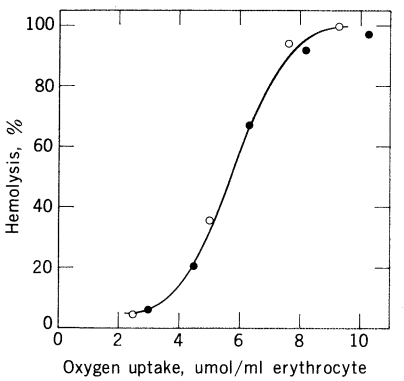

Fig. 3. Plot of percent hemolysis against oxygen uptake in the oxidation of erythrocytes of vitamin E-deficient rat (○) and control rat (●) at 37°C initiated with 74 mM AAPH.

under similar conditions (*5*). The rate of chain initiation in the oxidations shown in Fig. 1 is $2 \times 1.64 \times 10^{-7} \times 0.074 = 2.4 \times 10^{-8}$ mol radical/liter suspension/s. The rate of oxygen uptake ranged from 8×10^{-8} to 18×10^{-8} mol O_2/liter suspension/s and hence the kinetic chain length was obtained as 3.3 to 7.5, considerably larger than 1, suggesting that erythrocytes are oxidized by a free radical chain mechanism. This is probably the first experimental evidence that the biological membranes are oxidized by molecular oxygen by a free radical chain mechanism.

Figures 1 and 2 show that vitamin E, a potent antioxidant in the membrane (*8–10*), suppresses the peroxidation and also hemolysis of erythrocyte membranes. Interestingly enough, however, the plot of the extent of hemolysis as a function of oxygen uptake (Fig. 3) shows that the extent of hemolysis is determined primarily by the amount of oxygen uptake, that is, by the extent of oxidation

independent of the presence or absence of vitamin E. This implies that vitamin E suppresses hemolysis not physically but chemically by scavenging lipid peroxy radical and interrupting the chain reaction.

The hemolysis started even though a considerable amount (approximately one-third to one-half of the initial content) of vitamin E was remaining in the erythrocyte membranes. Vitamin E scavenges both peroxy radicals generated from AAPH ($AO_2\cdot$) which attack the membrane and lipid peroxy radicals ($LO_2\cdot$) which propagate chain reaction. However, vitamin E cannot completely suppress the oxidations of lipids and proteins and the hemolysis must take place when these oxidations proceed to a certain level. Further studies are apparently required to elucidate the detailed mechanisms of oxidative damage and hemolysis of erythrocyte membranes.

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