EFFECTS OF α-TOCOPHEROL AND ITS HOMOLOGS ON LUMINOL-DEPENDENT CHEMILUMINESCENCE INDUCED BY (Fe^{2+} + ASCORBATE) AND (Fe^{2+} + NADPH) IN RAT LIVER MICROSOMES

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The antioxidant function of the most widely used natural inhibitors of lipid peroxidation (LPO), namely the tocopherols, ubiquinones, and naphthoquinones, is due to the aromatic fragments of their molecules [1]. The antioxidant function of these substances is determined largely by their ability to react with free radicals, i.e., their antiradical activity. The number of carbon atoms in the isoprenoid chain of the molecule of 6-oxychromane and its derivatives with a differing length of their isoprenoid chain (6-oxychromanes – C1, C3, C6, C11), is virtually identical in homogeneous oxidation systems [2]. However, in regular and essentially microheterogeneous systems, especially in biomembranes, in which the effectiveness of interaction of the reagents is largely determined by their orientation and by their mutual accessibility, the antioxidant action of C16 (α-tocopherol) and its homologs differs sharply [3, 4]. Interaction of the chromophore luminol with hydroxyl and superoxide radicals is known to be accompanied by photoemission [5].

Inhibition of luminol-dependent chemiluminescence (LDCL) by various substances is thus frequently used to assess their antiradical activity. This method is suitable for both homogeneous and heterogeneous systems.

In this study the efficacy of action of 6-oxychromanes (C1, C6, C11, and C16) on photoemission of luminol and on accumulation of TBA-active LPO products, induced in microsomal suspensions, was compared.

EXPERIMENTAL METHOD

Male Wistar rats weighing 150-180 g were used. The microsomal fraction of rat liver was isolated by differential centrifugation. The protein concentration was measured by the biuret method, using bovine serum albumin as the standard. LPO and LDCL were induced, during continuous mixing, in medium containing: 0.1 M K,Na-phosphate buffer, pH 7.4 (37°C), 0.5 mg protein/ml, 0.25 mM NADPH or ascorbate, and 50 μM luminol. The LPO process and photoemission of luminol were induced by the addition of 10 μM $FeSO_4$. Chemiluminescence was recorded on a chemiluminometer, built in the Department of Physics and Biophysics, Medico-Biological Research Institute, Medical Academy, Sofia, Bulgaria. Accumulation of LPO products was determined from the quantity of malonic dialdehyde (MDA) formed [6].

α-Tocopherol (C16) was added to membrane suspensions by adding alcoholic solutions of these compounds in the necessary concentrations (final alcohol concentration did not exceed 0.5%). The 6-oxychromanes were inserted into the membranes during incubation of the substances for 10 min with the membrane suspensions at 37°C [7]. The following reagents were used: α-tocopherol ("Serva"), NADPH and ascorbate ("Reanal"), K_2HPO_4 and NaH_2PO_4 ("Merck"), 2-thiobarbituric acid, superoxide dismutase, and catalase ("Sigma"), TCA ("Reachim"), and luminol ("Koch-Light Laboratories Ltd."). The 6-oxychromanes (C1, C6, and C11) were synthesized in the Institute of Fine Chemical Technology, Academy of Sciences of the USSR (Moscow) [8].

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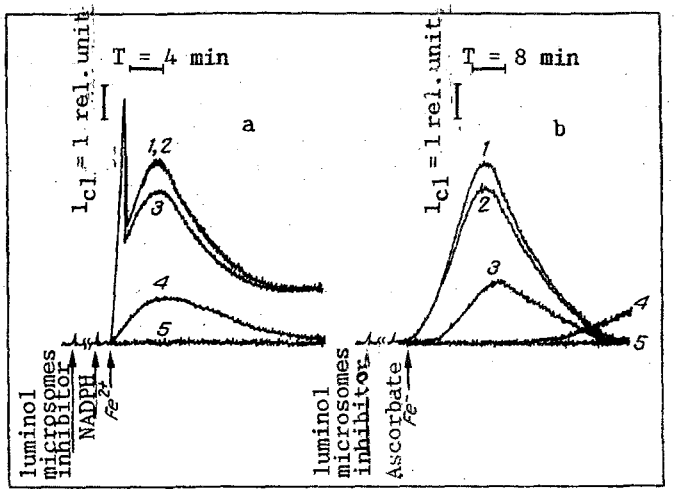

Fig. 1. Kinetic curves of luminol-dependent chemiluminescence induced in microsomal suspensions in presence and absence of 6-oxychromanes. a) (Fe^{2+} + NADPH); b) (Fe^{2+} + ascorbate). 1) Control; 2) C16; 3) C11; 4) C6; 5) C1. Conditions of incubation: 0.1 M K,Na-phosphate buffer, pH 7.4, 50 μM luminol, 0.5 mg protein/ml, 0.25 mM NADPH or ascorbate.

EXPERIMENTAL RESULTS

In the experiments of series I the efficacy of action of C16 and its homologs was compared on LDCL induced in microsomal suspensions. The choice of microsomal suspensions as a heterogeneous system was determined by two facts: the possibility of inducing LPO and LDCL by two methods, namely nonenzymic [(Fe^{2+} + ascorbate)-dependent] and cytochrome P-450-dependent [(Fe^{2+} + NADPH)-dependent]; b) the high index of unsaturation of the phospholipid composition of the microsomal membranes, which guarantees a high rate of free radical generation.

In the absence of luminol, and under the recording conditions which we chose, chemiluminescence was not observed after the addition of (Fe^{2+} + NADPH) or (Fe^{2+} + ascorbate) respectively to the incubation medium. The chemiluminescent response was recorded only after the addition of luminol and one of the chosen induction systems to the medium. The amplitude of the chemiluminescent response in the absence and in the presence of 6-oxychromanes in microsomal suspensions was determined in the experiments.

Figure 1 gives kinetic curves of luminol photoemission in microsomal suspensions during induction by a (Fe^{2+} + NADPH) system in the absence (curve 1) and in the presence (curves 2-5) of 6-oxychromanes. The kinetic curve in the absence of 6-oxychromanes is characterized by two phases: a) the rapid flash, recorded immediately (1-2 min) after addition of Fe^{2+} to the medium; b) slow flash (2-30 min) after the addition of Fe^{2+} to the medium, followed by flattening out on a plateau. The 6-oxychromanes C1 and C6 in a concentration of 100 μM effectively inhibited the chemoluminescent response. The inhibitory effect of C11 was weak compared with that of C6 and C1. C16 had no effect on luminol photoemission in a concentration of 100 μM.

Figure 2 shows the action of 6-oxychromanes on the amplitude of the slow flash of chemiluminescence (Fig. 2a) and accumulation of TBA-active products (Fig. 2b) during (Fe^{2+} + NADPH)-induced LPO in microsomal suspensions as a function of concentration. Clearly the inhibitory effect of the substances studied on chemiluminescence increased with an increase in the 6-oxychromane concentration and with a decrease in length of the hydrocarbon chain in the series C11 << C6 < C1. C16 (in concentrations up to 1 mM) had no significant action on chemiluminescence. Comparative analysis of the efficacy of action of the 6-oxychromanes on luminol photoemission and on accumulation of TBA-active LPO products in the incubation medium showed that C16 has virtually no effect on chemiluminescence and inhibits MDA accumulation. The short-chain C1 and C6 inhibited luminol photoemission and accumulation of TBA-active LPO products equally effectively.

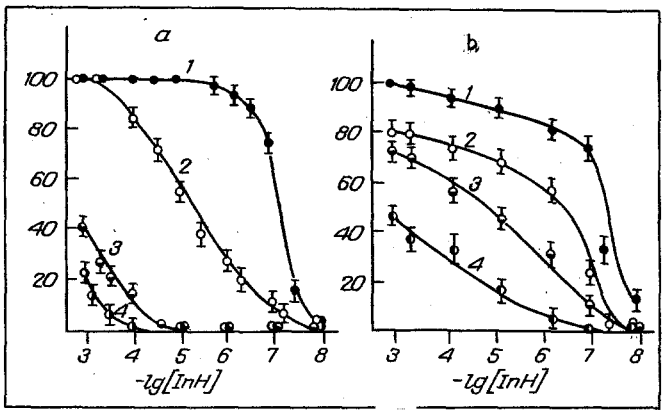

Fig. 2. Concentration dependence of action of 6-oxychromanes on amplitude of slow flash of luminol-dependent chemiluminescence (a) and accumulation of TBO-active LPO products (b), induced by (Fe^{2+} + NADPH) system in microsomal suspensions. Here and in Fig. 3, ordinate: a) inhibition of LDCL (in %), b) inhibition of accumulation of TBA-active LPO products (in %). 1) C1; 2) C6; 3) C11; 4) C16. Incubation conditions: 0.1 M K,Na phosphate buffer, pH 7.4, 50 μM luminol, 0.5 mg protein/ml, 0.25 mM NADPH, 10 mM Fe^{2+}. Incubation time 5 min, 37°C. MDA concentration in control was 5.1 nmoles/mg protein. InH) 100 μM 6-oxychromane.

In the next experiments the action of 6-oxychromanes was studied on LDCL induced by the (Fe^{2+} + ascorbate) system. The results of these experiments are given in Fig. 1b. In this case the chemiluminescent response included a slow flash, which was observed after a short lag period. The 6-oxychromanes C1, C6, and C11 affected the duration of the lag phase. The lag phase increased both with an increase in concentration of the inhibitors and with shortening of the length of the isoprenoid fragment of the chromane molecule (C11 < C6 < C1). C1 completely inhibited LDCL for 30 min. C6 and C11 sharply reduced the amplitude of the chemiluminescent peak. C16 (100 μM) had virtually no action either on the lag phase or on the amplitude of the chemiluminescent response.

Figure 3 shows the action of 6-oxychromanes on the amplitude of the slow flash of luminol photoemission (Fig. 3a) and on accumulation of TBA-active LPO products (Fig. 3b), induced by the (Fe^{2+} + ascorbate) system in membrane suspensions as a function of concentration. Clearly C1, C6, and C11 inhibited chemiluminescence. The inhibitory effect, moreover, increased in the order C11 << C6 << C1. Here, just as in the case of (Fe^{2+} + NADPH)-induced LDCL, C16 had no significant effect on photoemission (up to a concentration of 1 mM). Comparison of the curves reflecting the action of 6-oxychromanes on the amplitude of the chemiluminescent response and on accumulation of TBA-active LPO products as a function of concentration shows that: a) C1 and C6 inhibited photoemission and MDA accumulation equally effectively; b) C11 inhibited MDA accumulation more effectively than LDCL; c) C16 had virtually no effect on chemiluminescence, but it effectively inhibited accumulation of TBA-active LPO products in microsomal suspensions.

In the experiments of series II the effect of superoxide dismutase (SOD) and catalase on (Fe^{2+} + ascorbate)- and (Fe^{2+} + NADPH)-induced LDCL was studied. The experiments showed that SOD, in a concentration of 20 U/ml inhibited the first phase of (Fe^{2+} + NADPH)-induced chemiluminescence by 43% but did not affect (Fe^{2+} + ascorbate)-induced chemiluminescence. Catalase, in a concentration of 50 U/ml, inhibited the amplitude of the slow flash of (Fe^{2+} + NADPH)-induced chemiluminescence by 70% and of (Fe^{2+} + ascorbate)induced chemiluminescence by 75%. These findings suggest that during induction of luminol photoemission by the (Fe^{2+} + NADPH) system, superoxide radicals are formed in the phase of the rapid flash, whereas in the phase of the slow flash hydroxyl groups are formed, in agreement with data in the literature [5]. Incidentally, in Fe^{2+}-induced chemiluminescence at least two reactions are important: a) generation of hydroxyl radicals as a result of Fentonlike reactions. Interactions of these radicals with luminol induces photoemission; b) induction of chemiluminescence due to interaction of the superoxide radical with luminol.

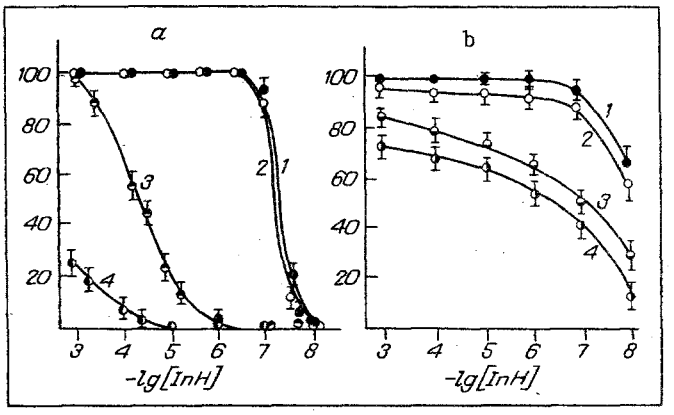

Fig. 3. Action of 6-oxychromanes on amplitude of luminol-dependent chemiluminescence (a) and on accumulation of TBA-active LPO products (b), induced by the (Fe^{2+} + ascorbate) system in microsomal suspensions, as a function of concentration. 1) C1; 2) C6; 3) C11; 4) C16. Conditions of incubation: 0.1 M K,Na-phosphate buffer, pH 7.4, 50 μM luminol, 0.5 mg protein/ml, 0.25 mM ascorbate, 10 μM Fe^{2+}. Incubation time 10 min, 37°C. MDA concentration in control 5.8 nmoles/mg protein.

For the first reaction the presence of substances reducing Fe^{3+} to Fe^{2+} in the system is essential. In the (Fe^{2+} + NADPH) system, in which the Fe^{2+} concentration is maintained by the superoxide generated in the system, photoemission is inhibited by SOD and catalase. In the (Fe^{2+} + ascorbate) system, in which the Fe^{2+} concentration is maintained chiefly by ascorbate, not by superoxide, luminol photoemission is inhibited by catalase and not inhibited by SOD. It can therefore be concluded that the LDCL which we recorded in the microsomal suspensions is due to hydroxyl and (or) superoxide radicals, and using both the (Fe^{2+} + NADPH) and the (Fe^{2+} + ascorbate) systems. Inhibition of LDCL gives information on the efficacy of interaction of the inhibitor with these radicals.

The experimental data examined above can be interpreted from two aspects: a) an increase in the efficacy of inhibition of LDCL and of MDA accumulation with shortening of the length of the hydrocarbon chain of the 6-oxychromanes; b) the higher efficacy of inhibition of LDCL and of accumulation of TBA-active LPO products by 6-oxychromanes during induction by the (Fe^{2+} + ascorbate) system than by the (Fe^{2+} + NADPH) system.

The first of these two facts can be explained on the grounds of a difference in the efficacy of interaction of 6-oxychromanes with radicals formed in the aqueous ($\cdot OH$, $O_2^{\cdot -}$) and lipid ($ROO\cdot$, $RO\cdot$, $R\cdot$) phases of the membrane suspension. We know that hydroxyl and superoxide radicals are initiators of free-radical oxidation chains of membrane lipids [9, 10]. Lipid radicals, in turn, participate in the lengthening and branching of chains [9, 10]. The experiments showed that luminol photoemission is the result of formation of hydroxyl and superoxide radicals in the incubation medium. We also know that MDA formation takes place through the generation of lipid radicals in membrane suspensions [9, 10]. Since C1 and C6 effectively inhibit both luminol photoemission and MDA accumulation, there is reason to suppose that they interact effectively with water-soluble and lipid radicals. C1 and C6 inhibit both the stage of initiation and the stage of lengthening and branching of the chain reactions. C16 does not affect luminol photoemission but inhibits accumulation of TBA-active LPO products. Consequently, C16 interacts only with lipid radicals, i.e., it acts only on the stages of lengthening and branching of the chains and cannot inhibit LPO in the induction phase. It can be concluded that with shortening of the length of the hydrocarbon chain of the 6-oxychromanes, the possibility of interaction of the substances with both lipid and water-soluble active forms of oxygen increases, and it is this which causes the increase in the inhibitory effect in the series C16 < C11 < C6 < C1.

Differences in the efficacy of the antioxidant action of the 6-oxychromanes can be explained by limitation of their mobility in the membrane with an increase in size of the isoprenoid fragment and as a result, by limitation of their access to centers of radical formation. We know that during (Fe^{2+} + ascorbate)-dependent induction of LPO, centers of generation of hydroxyl and superoxide radicals are located and uniformly distributed in the aqueous phase. During (Fe^{2+} + NADPH)-dependent induction of LPO they are located in the biomembrane, in the region of cytochrome P-450 reductase [11]. This explains the higher efficacy of inhibition of LDCL and the accumulation of TBA-active LPO products by 6-oxychromanes during induction by the (Fe^{2+} + ascorbate) system compared with the (Fe^{2+} + NADPH) system.

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