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ANTIOXIDANT BEHAVIOUR OF UBIQUINONE AND β-CAROTENE INCORPORATED IN MODEL MEMBRANES

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Experiments with model membranes, in which ubiquinone was incorporated, were performed in order to clarify the mechanism by which ubiquinone can prevent or control chain lipid peroxidation in biomembranes.

Comparing the behaviour of ubiquinone-containing vesicles with β-carotene containing vesicles we suggest that a possible explanation of the ubiquinone antioxidant effect could be to scavenge singlet oxygen and to affect structurally the lipid bilayer inhibiting hydroperoxide decomposition.

Key words: Ubiquinone, Carotene, Liposomes, Lipid peroxidation

Abbreviations used: UQ_3, ubiquinone-3; UQ_9, ubiquinone-9; UQs, ubiquinones; MDA, malondialdehyde.

INTRODUCTION

A major role for ubiquinone (UQ) and related lipophilic quinones is to transmit protons across membranes during an oxidation reduction cycle[1]. This function has been proposed as the basis for energy transduction in mitochrondria, chloroplasts and bacterial plasma membranes[2]. The details of quinone location in the membrane and the mechanism of electron transport in natural and model membranes has been the object of several investigations[3].

Besides its function as an electron carrier the reduced form of ubiquinone may also protect membrane phospholipids against peroxidation[4,5,6]. Studying enzymatic lipid peroxidation in the presence of NADH as an electron donor and of ADP-Fe^{3+} chelate in bovine heart mitochondria extracted and reconstituted either with UQ_{10} or UQ_3, we suggested that not only the reduced form of the quinones but also the oxidized one can behave as an antioxidant[7]. In order to clarify the mechanism by which UQ can prevent or control chain lipid peroxidation in biomembranes we performed experiments with

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model membranes in which UQ was incorporated and we have investigated the ability of these UQ-containing vesicles to resist lipid peroxidation.

The present data suggest that a possible explanation of the antioxidant effect could be to scavenge singlet oxygen and to affect structurally the lipid bilayer inhibiting hydroperoxide decomposition by metal catalysed reactions which promote spontaneous lipid peroxidation.

MATERIALS AND METHODS

Chemicals

Egg lecithin and phosphatidic acid were obtained from Lipid Products (Redhill, U.K.) and used without further purification. Diarachidonyl-L- α -lecithin (approx. 87%) was supplied by P.L. Biochemicals, G.m.b.H. (West Germany). α-tocopherol and β-carotene were purchased by Hoffman La Roche (Basel, Switzerland). Ubiquinones were a kind gift of Eisai Co., (Tokio, Japan). They were stored as a solution in absolute ethanol at $-20°C$ at a concentration of about 10 mM as determined by measuring the extinction decrease at 275 nm upon addition of KBH_4 (5 $mg.ml^{-1}$) and using an extinction coefficient of 12.25 $mM^{-1}.cm^{-1}$. All other chemicals used were of the highest available grade and were purchased from Merck (Darmstadt, West Germany) and Sigma Chemicals Co. (St. Louis, MO, U.S.A.).

Vesicle preparation

Phosphatidic acid (10 mol %) was added to egg lecithin and diarachidonyl-L- α -lecithin in chloroform: methanol (2:1 v/v) mixed in the proportions specified in the legends for figures and tables. When present, UQ, α-tocopherol and β-carotene, dissolved in chloroform were added to phospholipid solutions and the solvent was removed by evaporation under nitrogen. Aliquots of 50 mM Hepes (N-2-hydroxyethil-piperazine-N'-2-ethane-sulfonic acid) buffer, pH 7.4, were added to the dried lipid to give a phospholipid concentration of 3.2 $mg.ml^{-1}$ and an antioxidant concentration of 20–120 $nmol.ml^{-1}$.

The suspensions were sonicated with a probe sonicator (MSE) for 20 min at 4°C under a nitrogen atmosphere. The vesicle dispersions were then centrifuged for 15 min at 100,000 $\times$ g to remove any probe particles and large aggregates.

Determination of UQ, α-tocopherol and β-carotene incorporation

The amount of the quinones incorporated into bilayer vesicles was determined according to published method[8]. α-tocopherol and β-carotene incorporation was accomplished according to Dilley & Crane[9] and Liaanen-Jensen & Jensen respectively[10].

Autoxidation of lipid vesicles

Vesicles were left for a convenient number of days at room temperature under normal laboratory lighting in glass tubes and vortex-mixed each day. After the appropriate time intervals lipid peroxidation was measured.

Moreover, in vesicles freshly prepared lipid peroxidation was stimulated by iron salts. The reaction mixture, with a total volume of 1 ml, contained: phospholipid 0.2–0.4 mg; antioxidant as indicated in tables; $FeCl_2$ 0.3 mM. The pH of the final reaction mixture was 7.4; the reaction mixture was incubated at 30°C for 30 min. Lipid peroxidation was measured by the formation of thiobarbituric acid (TBA)-reactive material as malondialdehyde (MDA) at 532 nm[11]. Just prior to use 0.01 ml of 2% butylated hydroxytoluene (BHT) ethanolic solution was added to the TBA reagent. Lipid hydroperoxides were also measured on aliquots of vesicles with thiocyanate method according to Cavallini *et al*[12].

Lipid phosphorus determination was made according to Marinetti[13].

RESULTS

Previously we studied various methods to incorporate ubiquinones of different chain length in artificial membrane vesicles at amounts similar to that of mitochondrial and chloroplast thylakoid membranes[8], and then we evaluated antioxidative effect of ubiquinones on these phospholipid vesicles[14]. We showed that an efficient protection against autoxidation can be exerted by the various UQs about at the same extent independently of the side-chain length.

For this reason, this paper deals particularly with the antioxidative effect of UQ_3 that has often been found at the transition between the short- and long-chain quinones in its ability to reconstitute physiological electron transport[15,16] and which can be incorporated in controlled conditions into lipid vesicles[8].

In fact when we evaluated the UQ_3 incorporation into vesicles as a function of the amount of UQ_3 added we found (Fig 1A) that the relationship between the amount of the quinone added and its concentration in the hydrophobic phase is linear. In the same vesicles after 2 weeks we measured phospholipid autoxidation, which is a self-initiated autocatalytic process since polyunsaturated fatty acids are particularly prone to undergo air oxidation by a free radical chain mechanism.

The effect of different amounts of UQ_3 in monolamellar vesicles versus MDA formation is shown in fig. 1B. The results indicate that the amount of MDA, that is a non-specific but sensitive indication of the formation of lipid peroxides, decreases sigmoidically with increasing content of UQ_3 and that the concentration able to prevent 50% peroxidation is 13–14 nmol $UQ_3.mg^{-1}$ phospholipid. This is the amount normally incorporated in our previous study[14], and it approaches the physiological content of UQ in mitochondria and chloroplasts[16].

In Fig. 2 we report autoxidation of vesicles containing either the non physiological UQ_3 or the physiological UQ_9 in comparison with vesicles in which α-tocopherol was incorporated.

MDA formation plotted against the time course shows that the antioxidative effect is similar for both UQs, according to our previous data[14], and that after six days peroxidation is about 50% of control vesicles for UQ containing vesicles and 10% for α-tocopherol containing vesicles.

Ubiquinol showed the same antioxidant efficiency as α-tocopherol (data not shown).

Antioxidant properties of ubiquinones, as it can be seen in Fig. 2, are shown already during sonication; in fact MDA formation at 0 time is 6.8 nmol.mg^{-1} phospholipid in control vesicles, 3.6 nmol.mg^{-1} phospholipid in UQ containing vesicles

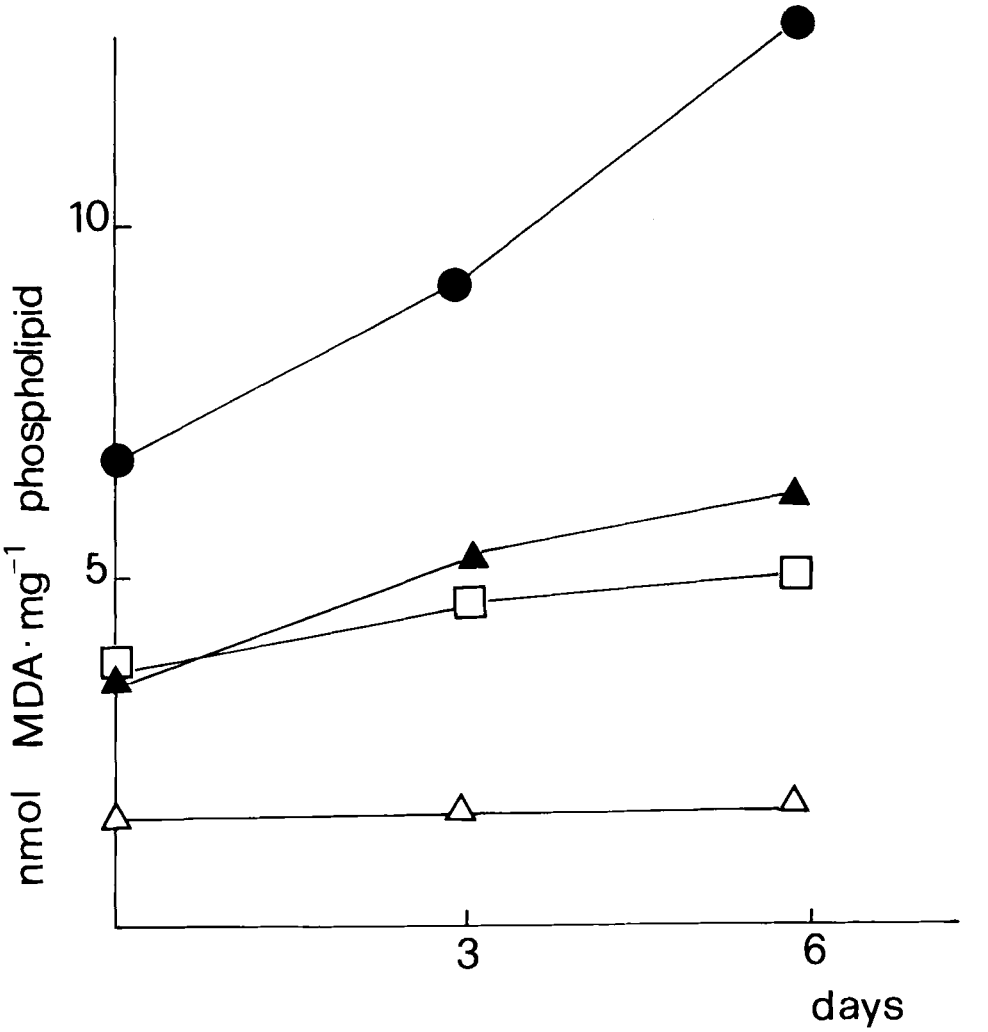

FIGURE 2 Effect of ubiquinone-3, ubiquinone-9 and α-tocopherol incorporated into vesicles on malondialdehyde formation versus time.
●—● Control vesicles; ▲—▲ UQ_9 containing vesicles; □—□ UQ_3 containing vesicles; △—△ α-tocopherol containing vesicles. UQ_9, UQ_3 and α-tocopherol incorporated were respectively 14, 14.3 and 13 nmol.mg^{-1} phospholipid.

and 1.5 nmol.mg^{-1} phospholipid in α-tocopherol containing vesicles. It is well known[17] that phospholipids, and lecithin in particular, may undergo, during the preparation of vesicles by sonication, oxidation of the unsaturated hydrocarbon chains. Sonic oscillation can give rise to superoxide radicals also in carefully controlled conditions and this is evident when sonication is performed in the presence of nitroblue tetrazolium a reagent able to detect $O_2^{\cdot-}$. In these conditions formazan production occurs during sonication with a parallel increase in absorbance at 560 nm (data not shown).

Dismutation of superoxide in the absence of superoxide dismutase may give singlet oxygen[18].

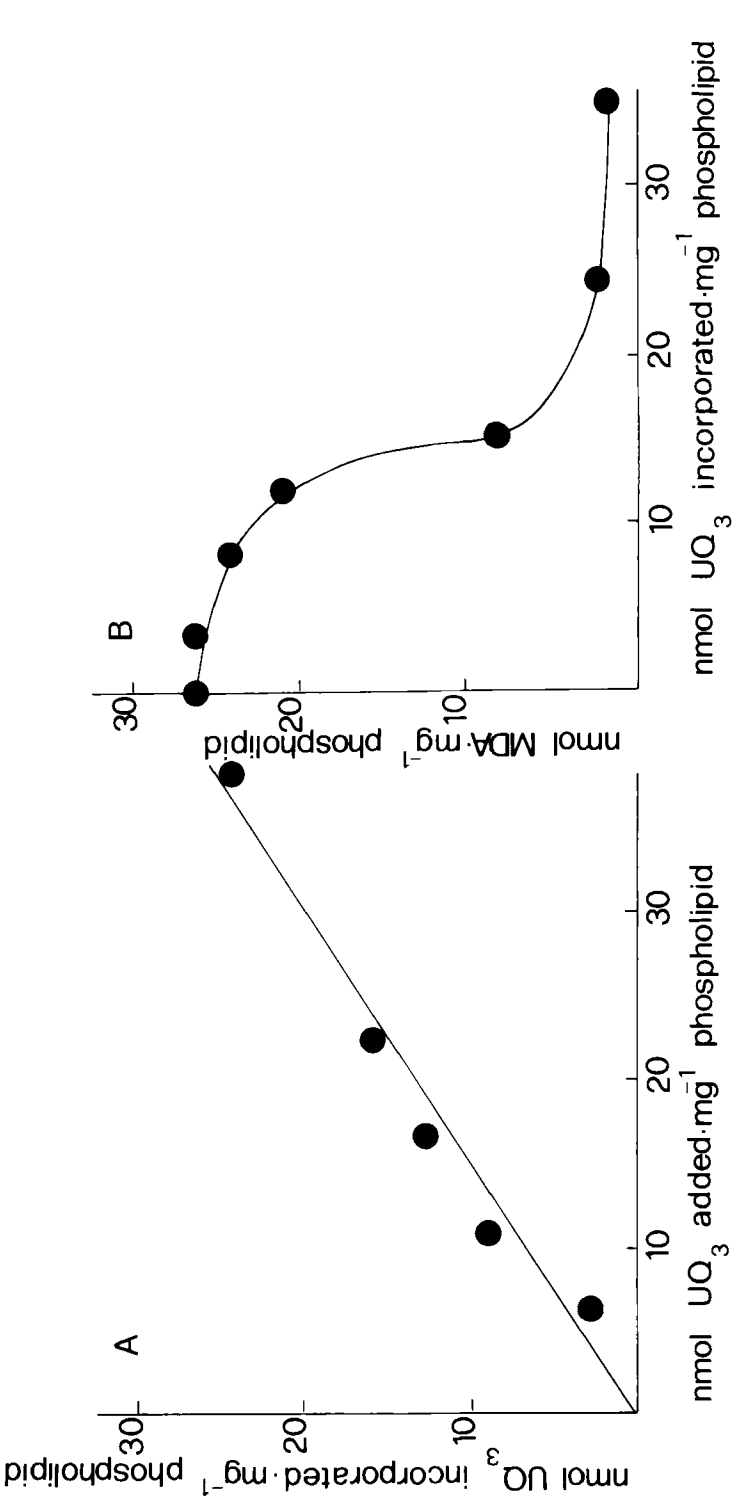

FIGURE 1 A. Effect of the amount of ubiquinone-3 added on the extent of incorporation into vesicles.
B. Effect of the amount of ubiquinone-3 incorporated into vesicles on malondialdehyde formation. For determination of UQ, malondialdehyde and total lipid concentration see Materials and Methods section.

TABLE I
Effect of ubiquinone-3 and β-carotene on lipid hydroperoxide and malondialdehyde formation during sonication.

Preparation of vesicles	malondialdehyde formation (nmol.mg^{-1} of phospholipid)	lipid hydroperoxide formation (nmol.mg^{-1} of phospholipid)
Control vesicles	5.0	157
UQ-containing vesicles	2.0	74
β-carotene-containing vesicles	1.5	50

Used vesicles contained 8% of arachidonic acid residues.
Lipid hydroperoxide content of the samples before sonication was 48 nmol.mg^{-1} of phospholipid. UQ and β-carotene incorporated were respectively 12 and 10 mol.mg^{-1} phospholipid.

TABLE II
Effect of ubiquinone-3 and β-carotene on Fe^{2+}-dependent lipid hydroperoxide and malondialdehyde formation.

Preparation of vesicles	malondialdehyde formation (nmol.mg^{-1} of phospholipid)	lipid hydroperoxide formation (nmol.mg^{-1} of phospholipid)
Control vesicles	31	1174
UQ-containing vesicles	4	676
β-carotene-containing vesicles	27	992

Vesicles preparations were obtained as described in the Material and Methods section and had the same composition as in Table I. The reaction mixtures were incubated at 30°C for 30 min.

Singlet oxygen was proposed to be the direct initiator of lipid peroxidation by a concerted addition-abstraction reaction with the diene bonds of unsaturated lipid giving rise to lipid hydroperoxides[19,20]. UQ_3 might react with $^1O_2$ and deactivate this molecule.

To assess this hypothesis we incorporated similar amounts of β-carotene, a well-known $^1O_2$ quencher[21] in vesicles having the same phospholipid composition. Table I reports hydroperoxide formation, which can be used as an estimation of the initial reaction of lipid peroxidation.

It appears that hydroperoxide formation in UQ- and β-carotene-containing vesicles immediately after sonication is similar, respectively 52% and 42% of control vesicles.

In the same experimental conditions MDA formation, that is one of the end products of lipid peroxide decomposition shows the same pattern.

It is well known that iron salts can stimulate lipid peroxidation by decomposing lipid peroxides to form alkoxyl and peroxyl radicals or by directly reacting with molecular 0_2 to produce $OH^{\cdot}$ or some species with similar reactive properties[22].

Table II shows the stimulatory effect of ferrous salt on hydroperoxide and MDA formation in the vesicles studied. In these conditions only UQ_3, and not β-carotene, is able to inhibit peroxidation stimulated by ferrous salt alone.

DISCUSSION

The data in Fig. 1B strengthen our previous reports[8,14] that UQ also in the oxidized form, is an effective antioxidant. The UQ content in phospholipid bilayer able to prevent 50% autoxidation corresponds to normal values of this quinone found in energy-transducing membranes, i.e. approximately 1 mol% relative to the lipid[16]. One possible explanation of the antioxidative effect of UQ could be to scavenge singlet oxygen as β-carotene. However, experimental results based largely on inhibition produced by $^1O_2$ scavengers need cautious interpretation since these compounds react with at least one organic peroxyl radical[23]. Furthermore, the direct demonstration that singlet oxygen participates in reactions is technically difficult. As far as the quenching of $^1O_2$ is concerned a recent paper indicates that UQ is an effective scavenger of $^1O_2$ as generated in microbicidal function of polimorphonuclear leucocytes[24].

Moreover UQ, but not β-carotene, may control peroxidation in the membrane inhibiting chain-branching reactions. Bivalent iron particularly, but also the oxidized form of the metal, may participate in chain branching significantly activating the development of lipid peroxidation reaction[22].

Ubiquinone, unlike ubiquinol and α-tocopherol, cannot donate a phenolic hydrogen atom to a free radical thus preventing autocatalytic free radical reactions, but it might probably stabilize membrane structure by physico-chemical interactions between UQ side chain and phospholipid fatty acyl chains. This close association with the polyunsaturated phospholipid molecules might inhibit diffusion and collision of free radicals, thus controlling the peroxidation rate and extent. A similar mechanism has been proposed for α-tocopherol by Lucy[25] on the basis of studies with model physico-chemical systems and could be extrapolated to our experiments in which an ubiquinone having a side chain length similar to that of α-tocopherol has been used. Interaction of a lipophilic molecule, such as UQ, with membranes modifies the molecular order and mobility of the lipid aliphatic chains. In fact fluorescence polarization of perylene is strongly enhanced by short-chain ubiquinones[26,27] which tend to intercalate molecularly within the lipid chains as also confirmed by calorimetric data[28]. Our findings in model membranes are not directly applicable to native membranes but would give useful information to clarify the antioxidative mechanism of ubiquinone if radicals were to arise particularly in mitochondria and Golgi apparatus where most of the quinone is distributed and where the presence of highly unsaturated fatty acids makes these membranes highly vulnerable to lipid peroxidation.

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