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Effects of free radicals produced by sonolysis on ubiquinone-containing vesicles

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The aim of this study was to evaluate both the effect of oxygen free radicals produced by ultrasonic irradiation on ubiquinone-3 incorporated into egg phosphatidylcholine vesicles and lipid peroxidation of the same vesicles. Owing to water sonolysis, degradation of polyunsaturated fatty acid residues occurred when model membranes were sonicated in the absence of ubiquinone. Under these conditions, Hepes buffer, but not formate, was able to protect against $HO^{\cdot}$ damage, probably because access to the site of $HO^{\cdot}$ formation is easier for the buffer. Incorporation of quinone prevented peroxidation due to $HO^{\cdot}$ radicals, with a concomitant decrease of ubiquinone content. Experiments performed to evaluate the effects of varying egg phosphatidylcholine concentration on conjugated diene formation and ubiquinone stability suggest that the antioxidant effect of this compound mostly resides in its capacity to act as a 'chain-breaker' antioxidant.

Introduction

When phospholipids are dispersed in the aqueous phase by ultrasonic irradiation, water molecules become the main source of the free radicals formed. In the sonolysis of water, the principal reactive species are the hydrated electron, the hydrogen atom and the hydroxyl free radical [1]

$$H_2O \rightarrow e^-_{aq},\ H^{\cdot},\ HO^{\cdot}$$

In the presence of oxygen, e^-_{aq} and $H^{\cdot}$ react at diffusion controlled rates to form univalently reduced oxygen, the superoxide anion and its protonated form, the perhydroxyl radical, both concurring by different pathways to the formation of H_2O_2 [2]. Owing to the reactive species formed, different redox reactions, which are often similar to the reactions produced by ionizing radiation [3], can occur during vesicle preparation by sonication, depending on phospholipids, buffers and other compounds present in the aqueous and/or lipid phases. In our research on the antioxidant activity of ubiquinones, we have shown that (i) sonication allows incorporation of comparable amounts of ubiquinone (Q) homologues independent of the length of the isoprenoid side chain [4]; (ii) antioxidant properties from ubiquinone-2 to ubiquinone-10 are similar [5]; (iii) they were already exhibited during the preparation of vesicles by sonication [6].

We recently studied the effect of oxygen-reactive species generated during ultrasonic irradiation on ubiquinones, both in the aqueous medium and in the phospholipid bilayer in the presence of Hepes (pH 7.2) [7]. The main product obtained after sonication of ubiquinone-0 aqueous solution was ubiquinol; some degradation also occurred, probably due to addition reactions of hydroxyl radicals with the aromatic ring structure of the quinone. When ubiquinone-3 (Q_3), chosen for its side-chain length similar to that of α-tocopherol, was incorporated into dimyristylphosphatidylcholine vesicles, hydroxyl radicals did not represent any potential danger for the antioxidant. Since Hepes was recently reported to be an effective scavenger of $HO^{\cdot}$ radicals generated by radiolysis of water [8], it became clear that the presence of this buffer could have complicated the interpretation of our previous results: Hepes, in fact, could have prevented the radical attack of Q molecules. The aim of this study was to reinvestigate the effect of sonolysis on ubiquinone-3 incorpo-

Abbreviations: Q_3, ubiquinone-3; DMPC, L-α-dimyristylphosphatidylcholine; EYPC, egg yolk phosphatidylcholine; LOOH, lipid hydroperoxides; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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rated into dimyristylphosphatidylcholine vesicles in the presence or absence of Hepes. We also studied the effect of the reactive species formed during ultrasonic irradiation on the quinone incorporated into egg phosphatidylcholine vesicles and the peroxidation of the same vesicles. The data reported may help clarify whether, under these experimental conditions, the quinone can behave as an antioxidant, directly reacting with either oxygen or lipid free radicals or with both.

Materials and Methods

Q_3 was a kind gift of Eisai, Tokio, Japan. Egg yolk phosphatidylcholine (EYPC) was obtained from Lipid Products (Redhill, U.K.) and was used without further purification. Its oxidation index, determined according to Klein [9], was 0.04. L-α-Dimyristylphosphatidylcholine (DMPC), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) were supplied by Sigma Chemical Company (St. Louis, MO, U.S.A.). Sodium formate was from Merck (Darmstadt, F.R.G.). All other chemicals used were of analytical grade. Stock solutions of EYPC or DMPC in chloroform and, when present, Q_3 in chloroform, were dried under nitrogen. The dried mixture was dispersed with a vortex mixer in 8 ml of double-distilled water or 0.01 M Hepes (pH 7.2) to give a final concentration of 4.2 mM for phospholipid and 60–70 μM for Q_3. The suspensions were then sonicated for different times up to 30 min under a nitrogen atmosphere with a Labsonic 2000 (B. Braun Melsungen AG, F.R.G.), according to a previously used procedure [7]. The power flow was 20 $\cdot cm^{-2}$ as measured by a calorimetric method. When the absorption spectra of Q_3 incorporated into vesicles was recorded, deoxycholate (pH 8.2) was added to vesicles to give a final concentration of 1% (w/v). Alternatively Q_3 was extracted from vesicle preparations according to the procedure described in Ref. 10 and analyzed by HPLC using an Altex ODS 5 μm column (15 × 0.46 mm). The column was eluted with water/methanol (9:1, v/v) containing 0.2% of 70% perchloric acid, at a flow rate of 1.5 ml/min. Detection was at 275 nm. Vesicles peroxidation was evaluated by different methods: (a) spectra in the wavelength range 320–215 nm were recorded and the increase in absorbance at 234 nm was taken as an indication of the appearance of conjugated dienes [9]. Sonicated samples were read against a blank containing the same amount of unsonicated vesicles. Deoxycholate (pH 8.2) was added before recording and the absorbance at 300 nm was taken as zero; (b) lipid hydroperoxides (LOOH) were measured with the thiocyanate method, according to Cavallini et al. [11]; (c) the fatty acid composition of phospholipid vesicles before and after sonication was determined by gas-liquid chromatography after methanolysis in acid/methanol [12]. The lipid phosphorus determination was made according to Marinetti [13].

Results

Firstly we reconsidered the efficiency with which Q_3 could react with oxygen radicals when it was within DMPC vesicles. Fig. 1 shows that in this case, contrary to what was reported in Ref. 6, $HO^{\cdot}$ radicals represented a danger for the antioxidant. In fact, after an irradiation time of 30 min, 60% degradation occurred for Q in the membrane environment. The scavengers, formate and Hepes were able to provide about 50% and total protection, respectively, against the absorbance decrease at 277 nm after a sonication time of 30 min. We then proceeded to investigate whether the ultrasonic effect on ubiquinone was different when this molecule was incorporated into phospholipid membranes with polyunsaturated fatty acid residues. At the same time, oxidative free radical damage to vesicles was examined. The most utilized method to measure the peroxidation in these experiments was based on the production of conjugated dienes. The ultraviolet spectra in the range of 320–215 nm of EYPC vesicles prepared in double-distilled water, after different times of sonication are shown in Fig. 2. It can be seen that as irradiation proceeded at constant ultrasonic intensity the formation of conjugated dienes increased considerably in control vesicles (Fig. 2A); on the other hand, the 234 nm peak was noticeably lower in Q-containing vesicles (Fig. 2B). The appearance of conjugated dienes involved, as a consequence, the formation of lipid hydroperoxides. These lipid peroxidation products or malonaldehyde have also been detected in model membranes subjected to different ionizing (γ-rays [14], X-rays [15], α-particles [16], electrons [17]) radiations. Fig. 3 shows that, under

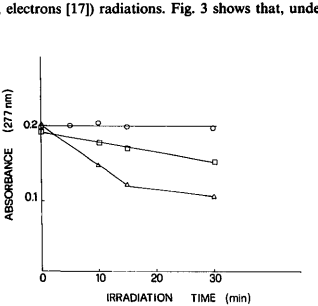

Fig. 1. Effect of the sonication medium on the time course of the absorbance change of ubiquinone-3 incorporated into DMPC vesicles: (○——○) 10 mM Hepes; (□——□) 50 mM formate; (△——△) double-distilled water.

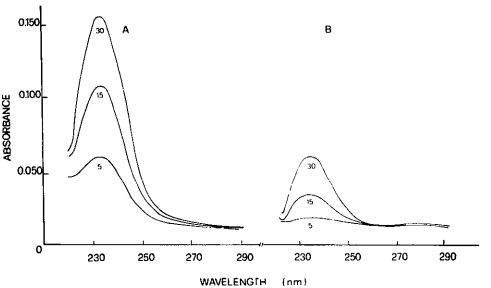

Fig. 2. (A) Sequential spectra of conjugated diene formation in EYPC vesicles exposed to sonication. (B) Sequential spectra of conjugated diene formation in Q_3-containing EYPC vesicles. The Q_3 concentration was 55 μM. The numbers indicate sonication time in minutes. Sonication was performed in double-distilled water.

the same experimental conditions, LOOH increased over at least 30 min sonication period in control vesicles, while their formation was very low in Q-containing vesicles. In Table I the percentage of the original amount of fatty acid residues in control vesicles and Q-containing vesicles after 30 min sonication in double-distilled water is reported. It can be observed that, in the absence of Q_3, the content of the polyunsaturated fatty acids with four, five and six double bonds decreases by 30%, 49%, and 57%, respectively, whereas in the presence of Q_3 there was only a slight loss of the total content of the same polyunsaturated fatty acids. The disappearance of polyenoic fatty acids led to a slight increase in the percent composition of saturated and monounsaturated fatty acids in control membranes.

The effect of different radical scavengers on the peroxidation of EYPC vesicles initiated by ultrasonic irradiation is reported in Table II. It can be observed that 30 min sonication performed in water led to an A_{234} value equal to 0.235, which decreased by 63% in Hepes and by 58% when ubiquinone was incorporated into the lipid bilayer. The determination of the amount of Q_3 evidenced that 25% of the quinone disappeared during the irradiation. α-Tocopherol, the phenolic antioxidant of choice, very efficiently prevented peroxidation, while the presence of formate was quite ineffective in preventing conjugate diene formation.

In order to assess which radicals, $HO^{\cdot}$, $HOO^{\cdot}$, lipid or lipid peroxyl, could have reacted with Q_3 and then degraded it, the experiments reported in Table III were

TABLE I

Fatty acid composition of control and Q-containing vesicles before and after sonication

The egg yolk phosphatidylcholine concentration was 4.2 mM. Vesicles were sonicated for 30 min in double-distilled water under the conditions described in Materials and Methods. Gas chromatographic analysis was performed according to the procedure described in Ref. 8. Data are the means of two analyses. The Q_3 concentration was 69 μM.

Fatty acid	Before sonication		After sonication			
	control vesicles		control vesicles		Q-containing vesicles	
	%	nmol	%	nmol	%	nmol
16:0	30.58±0.79	2569	32.14±0.31	2700	30.78±0.29	2585
16:1	2.22±0.01	186	2.35±0.05	197	2.25±0.01	189
18:0	12.41±0.47	1042	12.72±0.51	1068	11.96±0.10	1005
18:1	30.72±0.40	2580	32.70±0.68	2747	31.44±0.03	2641
18:2	13.85±0.28	1163	13.20±0.15	1109	13.95±0.07	1171
20:4($n-6$)	4.17±0.08	350	2.95±0.02	248	4.05±0.03	340
22:4($n-6$)	0.36±0.09	30	0.19±0.01	16	0.30±0.01	25
22:5($n-6$)	1.10±0.17	92	0.60±0.01	50	0.98±0.01	82
22:5($n-3$)	0.23±0.06	22	0.07±0.01	6	0.22±0.03	18
22:6($n-3$)	2.69±0.11	226	1.44±0.01	121	2.39±0.06	201

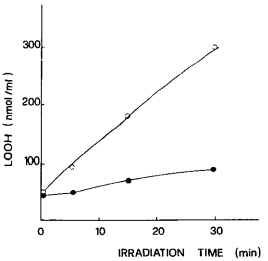

Fig. 3. Lipid hydroperoxide formation in EYPC vesicles (○——○) and in Q_3-containing EYPC vesicles (●——●) versus irradiation time. Sonication was performed in double-distilled water under the conditions described in Materials and Methods.

TABLE II

Effect of different radical scavengers on the peroxidation of EYPC vesicles induced by sonication

The egg yolk phosphatidylcholine concentration was 4.2 mM. Vesicles were sonicated for 30 min under the conditions described in Materials and Methods. Data are the means of three experiments with different vesicle preparations.

Addition to EYPC vesicles	A_{234}
None	0.235
50 mM formate	0.222
10 mM Hepes	0.088
0.06 mM Q_3	0.100
0.06 mM α-tocopherol	0.047

performed. This table shows the effects of changing the EYPC concentration on conjugated diene formation and Q stability in double-distilled water. As expected, A_{234} was enhanced by increasing the EYPC content in the vesicles. The amount of quinone incorporated into the lipid leaflets hardly changed when the phospholipid concentrations were 0.6 and 1.3 mM, while it decreased by 25% and 30% when vesicles were prepared with 4.2 and 10.5 mM, respectively.

TABLE III

Effect of phospholipid concentration on conjugated diene formation and Q_3 stability of EYPC vesicles

Vesicles were sonicated for 30 min in double-distilled water under the conditions described in Materials and Methods. Ubiquinone concentrations were determined by HPLC.

Vesicle preparation phospholipid (mM)	A_{234}		Q_3 concentration (nmol/ml)	
	$-Q_3$	$+Q_3$	before sonication	after sonication
0.6	0.080	0.000	66	62
1.3	0.100	0.030	65	64
4.2	0.235	0.096	69	52
10.5	0.380	0.152	69	47

Discussion

Our previous studies [6] to evaluate the direct effect of oxygen free radicals produced by ultrasonic irradiation on ubiquinone were always performed in the presence of Hepes, because of its efficient buffering capacity at near physiological pH. The data reported here show that in the absence of this buffer, $HO^{\cdot}$ radicals can attack ubiquinone even within the DMPC bilayer (see Fig. 1). In this sytem 10 mM Hepes acts as a scavenger more efficiently than 50 mM formate, probably because access to the site of $HO^{\cdot}$ formation is, perhaps, easier for the buffer. During ultrasonic irradiation, in fact, the radicals are produced in oscillating gas bubbles [18] and what is known from chemical studies on radiation in homogeneous solutions does not determine the efficiency of a scavenger for hydroxyl radicals produced sonolytically [19]. The rate constants of these radicals with the two scavengers are not very different, i.e., $2.7 \cdot 10^9\ M^{-1} \cdot s^{-1}$ with formate [20] and $5.1 \cdot 10^9\ M^{-1} \cdot s^{-1}$ with Hepes [8]; on the other hand, Hepes molecules, being less polar than formate, can be expected to enter the oscillating gas bubbles, where the radicals are produced, and thus to quench $HO^{\cdot}$ radicals more efficiently.

Experiments reported in Figs. 2 and 3 show that partial degradation of unsaturated hydrocarbon chains and formation of lipid hydroperoxides occur when model membranes are prepared by ultrasonic irradiation of polyunsaturated phospholipids. Peroxidation can be completely prevented, as previously shown by Köning [21], by adding a phenolic antioxidant, like α-tocopherol (see Table II). Also, incorporation of the oxidized form of ubiquinone into vesicles at a lipid/quinone molar ratio of 100:1.5, similar to that reported for phospholipids to ubiquinone in mitochondria [22], can prevent this oxidation. Protection is also provided in the presence of Hepes (see Table II), as expected by the high reaction rate constant of the buffer with the $HO^{\cdot}$ radical and for the reasons cited above. Thus, as was recently shown [23], the use of an appropriate buffer can lower the extent of peroxidation considerably during the preparation of model membranes by sonication. We cannot explain why formate was able to provide partial protection against the degradation of Q_3 in DMPC vesicles (see Fig. 1) and was unable to inhibit peroxidation of egg phosphatidylcholine vesicles (cf. Table II). When EYPC vesicles were sonicated in water, 330 nmol of polyunsaturated fatty acid were lost in control vesicles (cf. Table I) and the extent of ubiquinone decomposition in Q_3-containing vesicles was 25%, corresponding

to 15 nmol (cf. Table III). On a molar basis, it can be calculated that 20–30 molecules of polyunsaturated fatty acids are effectively protected by one molecule of oxidized ubiquinone. This ratio, much higher than 1, indicates that the oxidation proceeds by a free radical chain mechanism. The data reported in Table III can also help to determine whether Q protects polyunsaturated fatty acid residues inhibiting initiation by scavenging $HO^{\cdot}$ radicals, as Hepes does, or propagation by reacting with peroxyl radicals, as α-tocopherol does. The $HO^{\cdot}$ radical has to approach the phosphatidylcholine vesicle in order for the reaction to occur. On reaching these vesicles, there is a competition between polyunsaturated fatty acid and ubiquinone. The outcome of this competition will depend on the concentration of the two species and on the rate constants of $HO^{\cdot}$ radicals for the two species. Under our experimental conditions, the concentrations mostly used of polyunsaturated fatty acid and Q_3 were $1.72 \cdot 10^{-6}$ M and $6 \cdot 10^{-8}$ M, respectively. The k values of $HO^{\cdot}$ radicals for the reaction with linoleate ion and 9,10-antraquinone-2-sulfonate, obtained from pulse radiolysis in homogeneous solutions, were $1.1 \cdot 10^{10}$ $M^{-1} \cdot s^{-1}$ [24] and $5.6 \cdot 10^{9}$ $M^{-1} \cdot s^{-1}$ [25], respectively. We did not find a value for ubiquinone in the literature.

Had $HO^{\cdot}$ radicals attacked Q molecules, quinone degradation would have increased with decreasing phosphatidylcholine concentration, since the k values are not very different. This was not the case (see Table III): at low phospholipid concentration, almost no Q_3 depletion occurred. When the egg phosphatidylcholine concentrations were 4.2 and 10.5 mM, a 25% and 30% degradation of Q_3, respectively, took place, which corresponded to a 60% inhibition of peroxidation in both cases.

These data and the observation that one Q_3 molecule protects about 20–30 polyunsaturated fatty acid residues, suggests that the antioxidant effect of ubiquinone mostly resides in its ability to trap lipid peroxyl radicals, thus acting as a 'chain breaker' antioxidant. However, the molecular mechanism of this action still lacks an explanation, since ubiquinone, unlike ubiquinol and α-tocopherol, cannot donate a phenolic hydrogen atom to a free radical, preventing autocatalytic free radical reactions.

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