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ANTIOXIDANT ACTIVITY OF UBIQUINONE-3 IN HUMAN LOW DENSITY LIPOPROTEIN

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The ability of ubiquinone-3, a short chain ubiquinone homologue, to prevent Cu^{2+} induced oxidation of human low density lipoprotein was investigated. The results are as follows: in the presence of ubiquinone-3 the extent of peroxidation, as determined by the formation of thiobarbituric acid reactive substances, was only one third of that found in its absence; the quinone can also prevent the fragmentation of apolipoprotein B-100 and the increase of the net negative surface charge of the particle.

KEY WORDS: ubiquinone, low density lipoprotein, antioxidant, apolipoprotein B-100, Cu^{2+}-oxidation.

ABBREVIATIONS: LDL, low density lipoprotein; Q_3, ubiquinone-3; TBARS, thiobarbituric acid reactive substances; apo B-100, apolipoprotein B-100; BHT, butylated hydroxytoluene; PC, egg yolk lecithin.

INTRODUCTION

It has been suggested that free radical reactions may be involved in the development of atherosclerosis as a result of injury to blood vessel endothelial cells[1] and the oxidative modification of low density lipoprotein (LDL).[2] The mechanism of free radical mediated LDL modification and the pathological consequences deriving from it have recently been reviewed.[3,4] It has also been reported that the susceptibility to oxidation of LDL from individual subjects varies[5,6] and that these differences may be due to the presence of different lipophilic antioxidants mainly derived from dietary intake, such as vitamin E, β-carotene, retinyl ester[7] and flavonoids.[6] Furthermore, resistence to oxidative stress may also depend on differences in the content of peroxidizable lipids;[7] it is known that a high content of monounsaturated fatty acids protects lipoproteins against oxidative modification.[8]

A lipid-lowering drug, probucol, carried within the plasma lipoproteins inhibits the oxidative modification of LDL[9,10] and prevents the progression of atherosclerosis;[11] other antioxidants accumulating in lipoproteins might have similar protective effects.

A number of studies have reported the protective effect that the administration of ubiquinone (Q) has against oxidative tissue injury. It has been shown that ubiquinone

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is capable of reducing the cardiotoxicity of anthracycline antitumor antibiotics,[12] the peroxidative damage induced by treatment with CCl_4 and ethanol[13] and reperfusion injury.[14] Ubiquinone exists in all of the lipoprotein classes[15] and might also play a role in protecting lipoproteins against peroxidative damage. Previous studies have shown that, when incorporated into lipid vesicles by sonication, all of the Q homologues, from Q_2 to Q_{10}, have similar antioxidant properties.[16]

In this paper, we report the results of a study on the effectiveness of the Q_3 homologue in preventing copper catalyzed peroxidation in LDL and, for comparison, in lecithin vesicles. We have also investigated whether this molecule is able to prevent changes in the physico-chemical properties of LDL, including the increased electrophoretic mobility and the fragmentation of apolipoprotein B-100 which occur after Cu^{2+} oxidation.

MATERIALS AND METHODS

Egg yolk lecithin (PC) in $CHCl_3$-CH_3OH (2 : 1 v/v) was purchased from Lipid Products Ltd. (Redhill, U.K.). Ubiquinone-3 (Q_3) was a gift from the Eisai Co. (Tokyo, Japan). Stock solutions (10–20 mM) of Q_3 in absolute ethanol were stored at −20°C and standardized by the changes in UV absorption after $NaBH_4$ reduction. Reagent kits for total phospholipids were purchased from Biotrol (Paris, France). Nalgene filters (0.2 μm) were from Nalge Company (Rochester, New York, USA). LDL agarose gel electrophoresis was performed using the Paragon system supplied by Beckman (Brea, CA, USA). Aprotinin was from Sigma Chemicals Co. (St. Louis, MO). All other chemicals were from Merck (Darmstad, Germany), BDH (Poole, Dorset, U.K.).

Vesicle Preparation

Egg lecithin was dried to a thin film under a stream of nitrogen and the appropriate amounts of 0.01 M phosphate-buffered saline containing 10 μM EDTA (PBS-E) were then added to the lipid to give a phospholipid concentration of 3 mg/ml. The resulting suspension was vortex-stirred for 5 min and then sonicated under nitrogen for 10 min at 4°C, using a Labsonic 2000 sonicator.

In order to prepare Q_3-containing vesicles, stock solutions of quinone were dried in a test tube and PC vesicles (3 mg/ml) were added. The suspensions were vortex stirred for 10 min and then diluted to 0.3 mg PC/ml with PBS in order to obtain 7.5 μM, 30 μM and 60 μM Q_3.

Vesicle Oxidation

Q and non-Q containing vesicles were incubated with 10 μM $CuSO_4$ at 37°C. Control vesicles were also prepared in the presence of 0.2 mM EDTA. At different times oxidation was stopped by adding EDTA and BHT (final concentrations 2 mM and 20 μM, respectively). Peroxidation was evaluated by measuring thiobarbituric acid reactive substances (TBARS) at 532 nm against appropriate blanks.[17] All assays were performed in triplicate.

Isolation of LDL

Fresh plasma containing dipotassium EDTA (1.7 mg/dl) and aprotinin (90 kallikrein

inhibitory units/ml) was obtained from healthy non-smoker subjects. LDL (density 1.019–1.063) was isolated by preparative ultracentrifugation,[18] dialysed against at least four changes of PBS-E containing 0.1 mg/ml chloramphenicol at 4°C, and then sterilized by filtration and stored at 4°C. To prepare Q_3-treated LDL, stock solutions of Q_3 were dried under vacuum and LDL (1 mg phospholipid/ml) in PBS-E was added to give a final concentration of 0.1 mM Q_3; they were finally incubated by gently stirring at 37°C for 20 min.

Oxidation and Characterization of LDL

LDL (diluted with PBS-E up to 0.2 mg protein/ml) were incubated with 10 μM $CuSO_4$ at 37°C. Control-LDL (c-LDL) were also prepared in the presence of 0.2 mM EDTA without adding redox-active metals. At different times oxidation was stopped and peroxidation was evaluated by measuring TBARS as reported for vesicles. Protein was determined by the Lowry method[19] using bovine serum albumin as a standard and total phospholipids was quantified by means of enzymatic kits used according to the manufacturer's instructions. Agarose electrophoresis of LDL was performed using the Paragon Lipoprotein electrophoresis system. SDS-polyacrylamide gel electrophoresis was run in 5.75% acrylamide gels according to Laemmli.[20] LDL samples were delipidated according to Folch *et al.*[21] The protein was heated at 100°C for 5 min with 62.5 mM Tris buffer, pH 6.8, containing 5% SDS, 10% glycerol and 5% 2-mercaptoethanol. Aliquots containing 5–20 μg of protein were loaded on to the gels. The gels were stained with Coomassie Brilliant Blue R-250 in water/methanol/acetic acid (45 : 45 : 10) (v/v) and destained with the same mixture of solvents.

RESULTS AND DISCUSSION

The present work investigated the antioxidant activity of a representative ubiquinone on LDL oxidative modifications. Ubiquinone-3 was chosen because short isoprenoid chain Q homologues can be incorporated into phospholipid bilayers (and presumably into lipoproteins) by simply incubating the homologue adding it to the particles;[22] while physiologically long isoprenoid chain Q homologues can be efficiently incorporated into vesicles only by cosonication,[23] making it impracticable to incorporate such homologues into LDL. Our previous results have demonstrated that Q_3 has a protective effect against the vesicle peroxidation induced by ultrasonic irradiation,[24] iron salts[25] and an azoinitiator.[26] We have now extended the study by using copper salts in order to test the effect of different concentrations of Q_3 on 10 μM $CuSO_4$-induced peroxidation. The time-dependent oxidation of vesicles containing different amounts of Q_3 is shown in Figure 1A. After incubation, the extent of peroxidation in vesicles containing 7.5 and 30 μM Q_3, was respectively 64% and 30% of the quinone-free vesicles. Given that the rate and extent of autoxidation in vesicles containing 60 μM Q_3 were similar to those in vesicles containing 30 μM Q_3, these results show that Q_3 is also capable of inhibiting Cu^{2+} salts-induced lipid peroxidation. These findings are in agreement with the conclusion of Halliwell and Gutteridge,[27] who suggested that copper salts, as iron salts, cause the decomposition of the lipid hydroperoxides formed during the preparation of vesicles by sonication, to the alkoxyl and peroxyl radicals which are the true initiators of peroxidation.

To study the effectiveness of Q_3 as an inhibitor of LDL peroxidation, LDL solutions were incubated in the presence of 10 μM $CuSO_4$. Also in this case, Cu^{2+} may

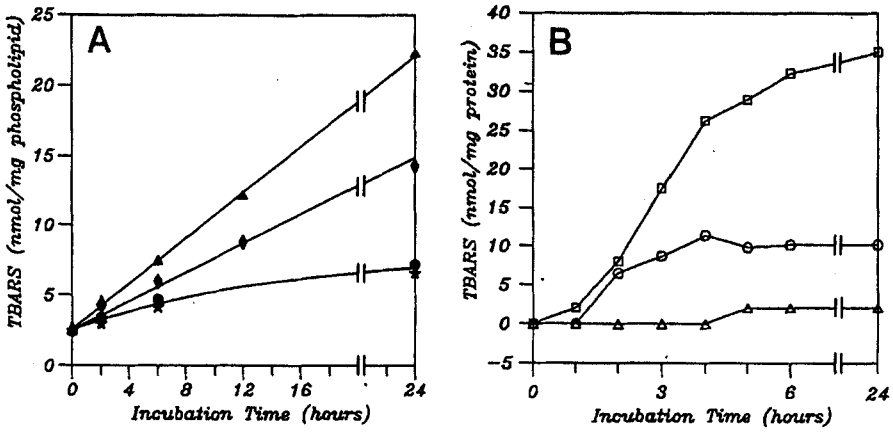

FIGURE 1 Antioxidant effect of Q_3 in lecithin vesicles (A) and in human LDL (B) incubated in the presence of 10 μM $CuSO_4$ for 24 h at 37°C. (A) vesicles contained 0 (▲), 7.5 (◆), 30 (●) and 60 (★) μM Q_3. (B) LDL samples were incubated with 0.2 mM EDTA (△), 10 μM $CuSO_4$ (□) and 10 μM $CuSO_4$ in the presence of Q_3 (○). At the indicated times aliquots were removed for the measurement of TBARS as described in Materials and Methods.

act by decomposing the small traces of lipid peroxides which are almost certainly present in freshly prepared LDL and whose existence is probably required to prime cell-free Cu^{2+}-mediated LDL oxidation.[28] The Cu^{2+} time-dependent oxidation of LDL is shown in Figure 1B. In control LDL, after a lag of about 1 h, there was a linear increase in the formation of TBARS during the following 3 h of incubation, which reached a value of 25 nmol/mg protein. After this time and until the end of incubation, the rate of production of TBARS moderately decreased. The delay of 1 h reflects the time during which endogenous antioxidants are depleted.[5-7] The largest quantity of TBARS formed during 24 h was 35 nmol/mg protein. The presence of EDTA completely prevented the formation of these end-products of lipid peroxide decomposition. In Q_3-LDL the contemporary presence of natural antioxidants and Q_3 completely inhibited the formation of TBARS during the lag period and the formation of lipid oxidation products increased only slowly up to 4 h (11 nmol/mg protein). After this time the content of TBARS remained constant until the end of incubation when, as with lecithin vesicles, the extent of peroxidation was only 30% that of the lipoprotein without quinone.

These results show that Q_3-containing vesicles and Q_3-LDL behave in a similar way. In the presence of Q_3, the oxidation rate is reduced but, because the amount of quinone present is too large to prevent the detection of the end of the inhibition period, antioxidant activity is found up to the end of incubation. These *in vitro* results can be explained on the basis of a recently proposed antioxidant mechanism of oxidized quinone.[26] It has been shown[29] that, when LDL is incubated in the presence of Cu^{2+} ions, agarose gel electrophoresis reveals a time-dependent increase in its mobility. The increased net negative surface charge is dependent on the binding of lipid peroxidation reactive products with positively charged amino groups of apo B-100 lysine residues.[5] Consequently, the effect of Q_3 on the changes in this physicochemical property of LDL was investigated. The time-dependent alteration of electrophoretic mobility after Cu^{2+} oxidation is shown in Table I. At the end of incubation,

TABLE I
Electrophoretic mobility of copper-oxidized LDL at different times of incubation[a]. Data are expressed in relation to the mobility of control samples

Treatment	time (h)	Relative Electrophoretic Mobility 0	3	6	24
0.2 mM EDTA (control)		1.00	1.00	1.00	1.00
10 μM $CuSO_4$		1.00	1.23	1.35	1.81
10 μM $CuSO_4$ + Q_3		1.00	1.19	1.15	1.31

[a] Samples were run on 0.5% agarose gel at pH 8.6 for 30 min.

the relative electrophoretic mobility of the oxidized samples was 1.81 in the absence of Q_3, and 1.31 in its presence. Thus, Q_3, acting as antioxidant, is able to decrease the anodic mobility of Cu^{2+} oxidized LDL.

Cu^{2+} LDL oxidation also leads to a degradation of apo B-100 as the result of a direct free-radical mediated peptide bond scission.[28,29] To follow the effect of Q_3 on the time course of the degradation of apo B-100 after copper-oxidation, the samples were subjected to SDS-PAGE under reducing conditions. The band of intact apo B-100 gradually decreased during incubation (Figure 2) and there was a concomitant appearance of fragments of lower molecular mass. After 24 h, apo B-100 completely disappeared, producing background smear fragments. In the presence of Q_3, the loss of intact apo B-100 was considerably reduced: only a slight decrease was evident after 4 h and, at the end of incubation, the band of apo B-100 was still evident and metal chelator EDTA had completely prevented its breakdown. Antioxidants such as butylated hydroxytoluene,[30] α-tocopherol[6] and the lipid-lowering drug, probucol,[9,10] have been shown to produce a marked decrease in the oxidative modification of LDL by scavenging lipid-derived radicals. The results described above suggest that also the

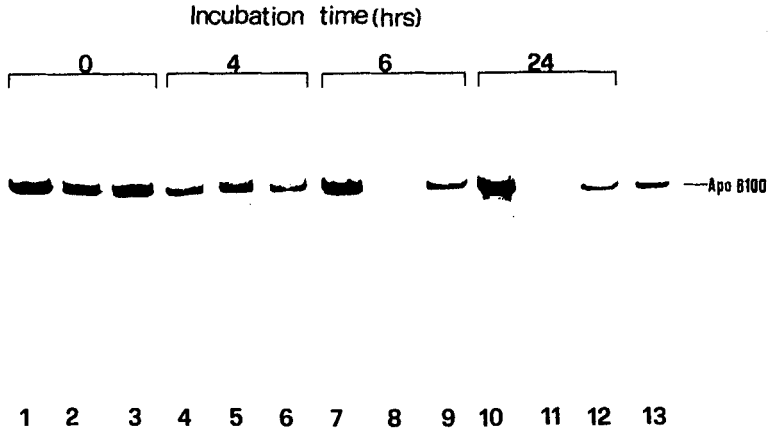

FIGURE 2 SDS-PAGE of copper-oxidized LDL. After different times of oxidation, LDL samples were delipidated and heated in sample buffer under reducing conditions. Samples (5–20 μg of LDL-protein) were run on SDS-PAGE as described in Materials and Methods. LDL controls (lanes 1, 4, 7, 10); LDL incubated with 10 μM $CuSO_4$ (lanes 2, 5, 8, 11); LDL incubated with 10 μM $CuSO_4$ in the presence of Q_3 (lanes 3, 6, 9, 12); non-treated LDL (lane 13).

antioxidant activity of Q_3, in both lipid vesicles and LDL, is related to its ability to trap lipid peroxyl radicals, thus accelerating the termination of lipid peroxidation.[25] Q_3 can also counteract the changes in the physico-chemical properties of LDL, since LDL peroxidation is accompanied by increased electrophoretic mobility and apo B-100 fragmentation. Recently, Stocker *et al.*[31] have shown that the reduced form of ubiquinone-10 is more efficient than α-tocopherol in inhibiting the human LDL oxidation initiated by azo-derivatives.

Ubiquinone is increasingly used as a therapeutic agent in a number of diseases involving the toxic effects of oxygen radicals.[32] In these pathological conditions, both the reduced and, albeit less efficiently, oxidized forms of Q may counteract the effects of oxidative damage by preventing the chain propagation of peroxidation. Moreover, it has also been shown that the use of compactin, an inhibitor of 3-hydroxy-3 methylglutaryl-coenzyme A reductase (the enzyme required for cholesterol biosynthesis), decreases Q levels in the LDL fraction[33] and that lovastatin lowers tissue levels of both cholesterol and Q in rats[34] and humans.[35] Since treatments that reduce hypercholesterolemia may impair both the bioenergetic role and the antioxidant function of ubiquinone, the administration of Q can, as it has recently been suggested,[35] be recommended in these patients.

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