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Vitamin K-antagonistic effect of plastoquinone and ubiquinone derivatives in vitro

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Abstract

Decyl-ubiquinone and decyl-plastoquinone were used as model compounds to test the potential effect of quinone derivatives on two enzymes of the vitamin K cycle in vitro. Substantial inhibition of γ-glutamate carboxylase was found, whereas vitamin K-epoxide reductase was inhibited to a much lesser extent. The inhibitory effect of both decylquinones was eliminated in a time-dependent way by solubilized microsomes, but not by purified carboxylase. Since a wide variety of prenylquinones occur as micronutrients, these results are of potential relevance for the effects of natural quinones in the human diet.

Key words: Plastoquinone; Ubiquinone; Phylloquinone; Menaquinone; Vitamin K

1. Introduction

Prenylquinones and prenylquinols like phylloquinone (vitamin K_1), plastoquinone-9 (PQ-9), ubiquinone-10 (UQ-10), and α-tocopherol (vitamin E) are produced by green plants where they are localized in cellular organs (Fig. 1). UQ-10 is a genuine constituent of the mitochondria whereas all the others occur in the thylakoid membrane, the photochemically active biomembrane of chloroplasts. Phylloquinone is a component of the photosynthetic electron transport within photosystem I [1,2], whereas PQ-9 and its corresponding quinol form a lipophilic redox system [3]. α-Tocoquinone and α-tocopherol represent another lipophilic redox system of the photochemically active thylakoids. The localization and function of these various compounds in the plant cell have been reviewed recently [4].

In man prenylquinones are part of the daily nutriture, and several members of this group are regarded as micronutrients. For instance vitamin K is an indispensable cofactor for the mammalian enzyme γ-glutamate carboxylase [5], a posttranslational enzyme which converts glutamate residues into γ-carboxyglutamate (Gla). Besides phylloquinone, also a number of bacterial menaquinones (generally known by their group-name vitamin K_2) have 'vitamin K activity'. The active cofactor for the carboxylase enzyme is vitamin K quinol (KH_2), which is oxidized into an epoxide (KO) during the carboxylation reaction. In two successive steps KO may subsequently be reduced via the quinone into KH_2, so that it may be re-used several thousand fold. The physiological cofactor for the reductase(s) involved in the recycling of vitamin K is still unknown, for in vitro systems dithiols (dithiothreitol, thioredoxin) have proven to be efficient [6].

Except for vitamin E, which is a weak inhibitor of carboxylase [7], no information is available concerning the in vivo or in vitro interaction of prenylquinones with the vitamin K-dependent carboxylase is available at this time. Here we demonstrate that the synthetic derivatives decyl-plastoquinone (d-PQ) and decyl-ubiquinone (d-UQ) may significantly affect the bovine liver vitamin K-dependent carboxylase. It is suggested that also natural (dietary) prenylquinones should be evaluated for their possible effects on human vitamin K status.

2. Materials and methods

2.1. Materials

Phylloquinone (vitamin K_1), menaquinone-4 (MK-4), Triton X-100, dithiothreitol (DTT), 3-((cholamidopropyl)-dimethylammonio)-1-propane sulphonate (CHAPS), d-PQ, d-UQ, and L-α-phosphatidyl choline from egg yolk were from Sigma (St. Louis, MO). Before use d-PQ and d-UQ were purified further by high performance liquid chromatography using a reversed phase column (Chrompack, Lichrosorb RP-18) and isocratic elution with 70% (v/v) methanol in H_2O as an eluent. The various quinones were converted to the corresponding quinols by incubating 6 mM of quinone in the presence of 150 mM DTT at pH 8.5 and 37°C overnight in a light-protected tube. KO was prepared according to the method of Tishler et al. [8]. The pentapeptide Phe-Leu-Glu-Glu-Leu (FLEEL) was purchased from Vega Biochemical Co. (Tucson,

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AZ), $NaH^{14}CO_3$ (56 Ci/mol) and Formula 989 from New England Nuclear (Dreieich, Germany). Salt washed microsomes were prepared from normal bovine liver according to earlier described methods [9], and were used as starting material for the purification of carboxylase according to the method of Wu et al. [10]. All chemicals were of analytical grade or better.

2.2. *Carboxylase assay*

Standard reaction mixtures (0.125 ml) contained: 1 mg of microsomal proteins, 0.4% (w/v) CHAPS, 0.5 M NaCl, 25 mM Tris-HCl (pH 7.5), 4 mM FLEEL, 1 M $(NH_4)_2SO_4$, 1.5 μCi $NaH^{14}CO_3$, 4 mM DTT, and 200 μM of either vitamin K, KO or KH_2. Inhibitors were added as indicated. For tests with purified carboxylase 2 μg of enzyme was incubated in 0.125 ml reaction mixtures containing 0.1% (w/v) phosphatidyl choline, 0.1% (w/v) CHAPS, 0.15 M NaCl, 50 mM Tris-HCl (pH 7.5), 4 mM FLEEL, 1.5 μCi $NaH^{14}CO_3$ and 200 μM of vitamin KH_2. Standard incubations were performed for 15 min at 20°C for washed microsomes and 30 min at 20°C for purified carboxylase. The reactions were stopped as detailed earlier [11]. All data are presented as the means of three independent experiments.

2.3. *KO-reductase assay*

Standard reaction mixtures (0.25 ml) contained 2 mg of microsomal proteins, other components were as described for the carboxylase assay except for FLEEL and $NaH^{14}CO_3$, which were omitted. Extraction and analysis of the samples were performed according to Thijssen [12].

2.4. *Analytical methods*

Protein concentrations were determined according to the procedure described by Sedmak and Grossberg [13].

3. Results

In this paper vitamin K activity is defined as the ability of a compound to function as a cofactor in the CO_2-fixation in an in vitro model system containing bovine liver microsomes, the pentapeptide FLEEL, and $NaH^{14}CO_3$. In this system we have tested the vitamin K activity of d-UQ and d-PQ both in their quinone and their quinol form. The data are given in Table 1 and demonstrate that neither of these compounds had vitamin K activity. Vitamin K inhibitory was tested in a similar system, to which 0.2 mM K_1 quinone had been added. In this way we found substantial vitamin K_1 antagonistic activity of both d-UQ and d-PQ in their quinol as well as in their quinone form. The inhibition as measured in the carboxylase assay (Table 1, 2nd lane) was more pronounced than that in the KO-reductase assay (Table 1, 3rd lane). Since carboxylase and KO-reductase are two distinct enzymes with different functions in the vitamin K cycle, these data indicate that carboxylase is the prime target enzyme for both inhibitors.

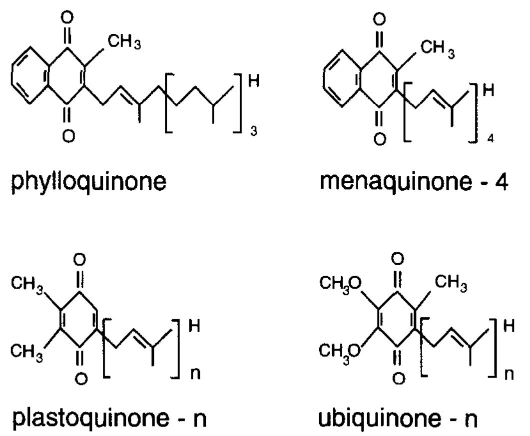

Fig. 1. Structure formulas of common prenylquinones. For natural plastoquinones and ubiquinones *n* represents the number of isoprene residues, which may vary depending on their origin. In the synthetic model compounds used in this paper, the 3-position of the quinones is occupied by an unbranched saturated decyl chain.

In a second set of experiments we have tried to estimate the inhibitor concentration required for half-maximal inhibition. Incubations were performed under standard conditions and varying inhibitor concentrations using 200 μM of either phylloquinone or menaquinone-4. A typical example of the inhibition curves thus obtained is given in Fig. 2, where we have represented the inhibition of various forms of phylloquinone by d-UQ. It is noteworthy that the extent of inhibition was independent of the reduction state of the vitamin, which indicates that among the enzymes of the vitamin K cycle, γ-glutamylcarboxylase is the one most strongly inhibited

Table 1
Effects of prenylquinones on the enzymes of the vitamin K cycle

Compound added	Concentration (mM)	Cofactor activity for: carboxylase (pmol $CO_2 \cdot min^{-1}$)	Vitamin K-antagonistic activity for:	
			carboxylase (% inhibition)	(% inhibition)
K_1H_2	0.2	100	n.d.	n.d.
K_1	0.2	68	0	n.d.
KO	0.2	52	n.d.	0
d-PQ	1	0	76	11
d-PQH_2	1	0	78	n.d.
d-UQ	1	0	80	10
d-UQH_2	1	0	81	n.d.

The cofactor activity for γ-glutamylcarboxylase was measured in the crude microsomal system and is expressed as pmol CO_2 incorporated per minute. Vitamin K-antagonistic activity was tested: (a) in a carboxylating system containing a fixed amount of vitamin K_1 quinone (0.2 mM), and (b) in the KO-reductase system; the data are expressed as the percentage to which both systems are inhibited. All data are the means of triplicate experiments. n.d. = not determined.

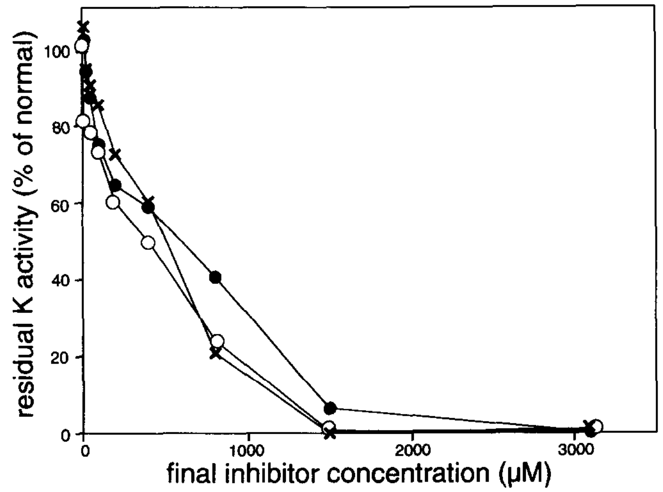

Fig. 2. Inhibition of the vitamin K-dependent carboxylation by d-UQ. The reaction was performed with salt-washed microsomes, and was started with 0.2 mM of either vitamin K_1H_2 (●), vitamin K quinone (○) or vitamin KO (×). 100% residual vitamin K activity stands for 15 (for KH_2), 10.5 (for K), and 7.5 (for KO) pmol CO_2 fixed per min, respectively.

by d-UQ and d-PQ. This is consistent with the data obtained in Table 1, where – in a direct test – at similar concentrations of d-UQ and d-PQ the inhibition of KO-reductase (lane 3) was 7–8 times less than that of carboxylase (lane 2); 10–50 times higher inhibitor concentrations were required for blocking KO-reductase to the same extent as carboxylase (data not shown). In Fig. 2 half-maximal inhibition was found at about 400 µM of inhibitor; at 4-fold higher inhibitor concentration the carboxylase reaction was almost completely blocked.

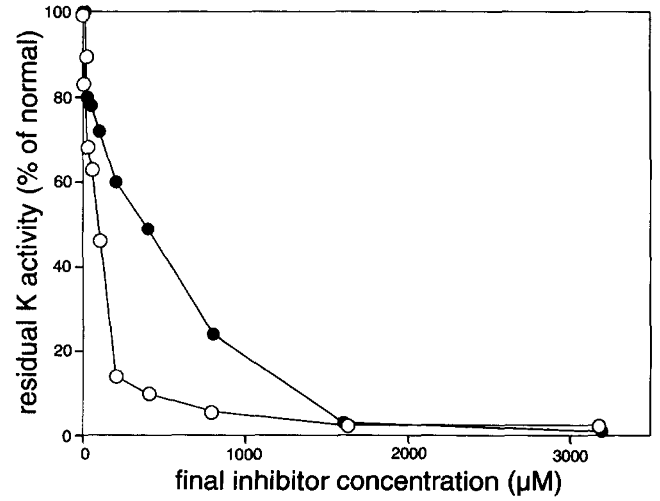

Fig. 3. d-UQ inhibition in salt-washed microsomes and purified carboxylase. Vitamin K_1H_2 (0.2 mM) was used as a coenzyme. Results are presented as percentage of the non-inhibited reaction. Symbols: (●) salt-washed microsomes; (○) purified carboxylase. 100% residual vitamin K activity stands for 15 pmol CO_2 fixed per min in the microsomal system and for 1.9 pmol CO_2 fixed per min by purified carboxylase.

From these curves inhibitor concentrations required for 50% inhibition were calculated for all combinations, and all were closely similar: 470 µM and 390 µM for d-PQ and d-UQ in the phylloquinone system, and 460 µM and 450 µM for d-PQ and d-UQ in the menaquinone system, respectively. So it seems that both menaquinone and phylloquinone are antagonized by d-UQ and d-PQ, and that the antagonistic effects of both compounds are of the same order of magnitude.

Recently a purification procedure for carboxylase has been described [10]. Preparations thus obtained are devoid of KO-reductase activity. We have also measured the sensitivity of purified carboxylase for d-UQ, and the results are shown in Fig. 3. It turned out that in the purified system 50% inhibition was obtained at 5-fold (91 vs. 430 µM) lower inhibitor concentrations. For d-PQ these figures were 85 and 426 µM, respectively (data not shown).

Finally we have prepared a time course of the inhibited and the non-inhibited reactions. As is shown in Fig. 4A the non-purified system is inhibited by d-UQ (and by d-PQ, data not shown) for 10 min, after which the carboxylation reaction proceeds at a normal rate. Preincubation of the inhibitor with the microsomal proteins for 15 min prior to starting the carboxylation reaction with

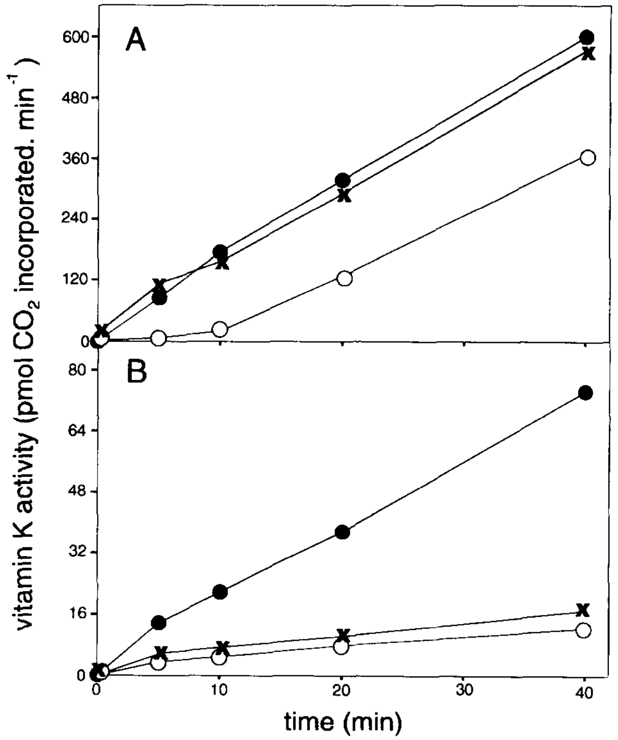

Fig. 4. Time-dependent inhibition of the carboxylation by d-UQ. Vitamin K_1H_2 (0.2 mM) was used as a coenzyme, and all reactions were performed at a fixed inhibitor concentration (1 mM). (A) Salt-washed microsomes. (B) Purified carboxylase. Symbols: (●) non-inhibited reaction; (○) carboxylation in the presence of inhibitor; (×) carboxylation after preincubation of inhibitor and microsomal proteins for 15 min at 20°C.

vitamin K even completely abolished the inhibitory effect. Apparently the inhibitor was degraded or removed from solution during the preincubation period. As is shown in Fig. 4B purified carboxylase was unable to neutralize the inhibitory effect of d-UQ even after incubation periods of 40 min and longer.

4. Discussion

A wide variety of quinone derivatives form part of the human diet, whereas our knowledge about the intestinal absorption and pharmacology of these compounds is still far from complete. Also it is unknown if, and to what extent, prenyl- and other quinones interfere with vitamin K-dependent reactions in liver, bone, and other tissues known to contain γ-glutamate carboxylase. To get an impression about the potential vitamin K or vitamin K-antagonistic activity of these quinones, we have used bovine liver in vitro systems, in which two synthetic derivatives plastoquinone and ubiquinone were tested.

It was found that both model compounds (decyl-plastoquinone and decyl-ubiquinone) behaved very similarly. Salt-washed, solubilized microsomes were used to compare the effects on γ-glutamate carboxylase and vitamin KO-reductase. Both quinones turned out to be inhibitors of the enzymes of the vitamin K cycle, but the effect on carboxylase was much stronger than that on KO-reductase. A problem here is that KO-reductase has not yet been purified, so that the experiments had to be performed in the rather crude microsomal system. It cannot be excluded, therefore, that part of the inhibitory activity is masked by non-specific adsorption of the decylquinones to contaminating proteins or phospholipids. This became clear from an experiment in which we compared the inhibitory activity of d-UQ in solubilized microsomes and purified carboxylase. It turned out that the purified enzyme was inhibited at least 5-fold stronger than the non-purified one.

An interesting phenomenon was that in time-course studies in the microsomal system the inhibitory effect of decylquinones decreased after 10–15 min, whereas in the purified system the inhibitory activity persisted, also after very long incubation periods. Pre-incubation of d-UQ with the solubilized microsomes even completely eliminated its inhibitory effect. Whether this elimination forms part of a biochemical degradation pathway is not clear at this time, but if so this pathway must be able to select plastoquinone and ubiquinone derivatives from phylloquinone and menaquinones, because the latter compounds are not affected under the conditions employed.

From the data presented in this paper we conclude that derivatives of plastoquinone and ubiquinone may form a new class of vitamin K-antagonists. It seems plausible that also natural quinones (e.g. those prominently found in the human diet) may possess vitamin K antagonistic activity in vitro. If similar effects will be found in vivo, dietary quinones may interfere with vitamin K-mediated processes like blood coagulation and bone metabolism.

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