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The Potent Antioxidant Activity of the Vitamin K Cycle in Microsomal Lipid Peroxidation

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ABSTRACT. In the vitamin K cycle, vitamin K-hydroquinone, the active cofactor for γ-glutamylcarboxylase, is continuously regenerated. The successive pathways contain oxidation of the hydroquinone to the epoxide, followed by reduction to the quinone and reduction to the hydroquinone. Vitamin K-hydroquinone is a potent radical scavenging species (Mukai *et al.*, *J Biol Chem* **267:** 22277–22281, 1992). We tested the potential antioxidant activity of the vitamin K cycle in lipid peroxidation reactions (thiobarbituric acid reactive substances, TBARS) in rat liver microsomes. As prooxidant we used Fe^{2+}/ascorbate, NADPH-Fe^{3+}/ATP, and NADPH/CCl_4. Vitamin K (≤50 μM) on its own did not influence the formation of TBARS. In combination with 1 mM dithiothreitol (DTT), the reductive cofactor for the microsomal enzyme vitamin K epoxide reductase, vitamin K suppressed lipid peroxidation with a concentration that blocked the maximal response by 50% (IC_{50}) of ca. 0.2 μM. Vitamin K_1 (phylloquinone) and vitamin K_2 (menaquinone-4) were equally active. Warfarin (5 μM) and chloro-vitamin K (50 μM), inhibitors of vitamin K epoxide reductase and γ-glutamylcarboxylase, respectively, were able to completely abolish the antioxidant effect. Lipid peroxidation was inversely related to the amount of vitamin K hydroquinone in the reaction. Vitamin K epoxide reductase seemed sensitive to lipid peroxidation, with half of the activity being lost within 10 min during oxidation with NADPH/CCl_4. The inactivation could be attenuated by antioxidants such as vitamin E, reduced glutathione, and menadione and also by a K vitamin in combination with DTT, but not by superoxide dismutase and catalase. The results show that the vitamin K cycle could act as a potent antioxidant, that the active species in all probability is vitamin K-hydroquinone, and that the primary reaction product is the semiquinone. The results also show that the reaction product is processed in the vitamin K cycle to regenerate vitamin K-hydroquinone. BIOCHEM PHARMACOL **54**;8:871–876, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. antioxidant; lipid peroxidation; vitamin K; vitamin K cycle; vitamin K epoxide reductase

It is well recognized that biological membranes are vulnerable to peroxidation reactions. Lipid peroxidation is initiated by free radicals originating mainly from oxygen and oxygen-derived species. Lipid peroxidation may disturb membranous integrity, causing cellular disfunction or even cellular death [1–3]. Biological membranes are mainly protected against lipid peroxidation by the fat-soluble antioxidant vitamin E, probably in combination with recycling activities [4–6]. In addition to vitamin E, numerous other food constituents such as carotenes [7], flavonoids [8], phenoles [9], and quinones [10–12] may protect against lipid peroxidation; indeed, prevention of lipid peroxidation can be demonstrated in *in vitro* experimental systems. Some of these compounds, such as the benzohydroquinones ubiquinol [13] and plastoquinol [14], may additionally exert potent antioxidant activity by regenerating vitamin E from its radical (α-tocopheroxyl radical).

The K vitamins are fat-soluble compounds essential for the posttranslational conversion of protein-bound glutamates into γ-carboxyglutamates in various target proteins, including the clotting factors [15]. The 1,4-naphthoquinone structure of the K vitamins resembles the benzoquinone structure, and thus these vitamers may contain antioxidative properties as well. Generally, menadione is used as the prototype of vitamin K. Menadione is a very potent inhibitor ($IC_{50} \ll 5$ μM) of microsomal lipid peroxidation. Its activity is believed to result mainly from its capacity to divert electrons from membrane lipids [12, 16]. Active K vitamins differ, however, from menadione by a poly-isoprenoid (menaquinones or vitamin K_2) or a phytyl (phylloquinone or vitamin K_1) chain at the 3 position. Talcott *et al.* [18] showed that 3-substitution weakened the activity of menadione as an inhibitor of lipid peroxidation. Furthermore, the water insolubility of the K vitamins may prevent them from catching electrons. Indeed, studies from several groups showed only weak activity ($IC_{50} \gg 100$ μM) of K vitamins as inhibitors of lipid peroxidation in various test systems [16–19]. On the other hand, the hydroquinone of vitamin K_1 may act as a potent antioxidant. Mukai *et al.* found the hydroquinone to be 10 times as potent as α-tocopherol in the reaction with phenoxyradicals [20] and

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100 times as potent as ubiquinol in regenerating vitamin E from its radical [14].

Vitamin K-hydroquinone, in fact, is the active cofactor of the enzyme γ-glutamylcarboxylase. Coupled to the carboxylase reaction, the hydroquinone is oxidized to vitamin K epoxide. Regeneration of the hydroquinone is warranted by vitamin K epoxide reductase, which reduces the epoxide to the quinone and thereafter to the hydroquinone [21]. The true mechanism of how the oxidation of vitamin K-hydroquinone leads to the γ-proton abstraction of glutamate is not yet completely clear [22], although it has been suggested that the semiquinone radical may act as intermediate in the reaction [23–25]. The notion that this vitamin K cycling may accommodate potent antioxidant activity is supported by the fact that 1) epoxidation can be dissociated from carboxylation and 2) that microsomal vitamin K epoxide reductase is present in reserve [26, 27]. Here, we report on experiments showing a potent inhibitory effect of the vitamin K cycle on microsomal lipid peroxidation.

MATERIALS AND METHODS

Microsomal Preparations

Microsomes were prepared from the livers of male Wistar rats (ca. 250 g) fed normal rat chow. Tissue homogenization was in 0.02 M Tris, 0.15 M KCl, 0.25 M sucrose buffer, pH 7.4 (tissue/buffer, 1/3). Microsomes were sedimented from the 18,000 × *g* supernatant by centrifugation at 105,000 × *g* for 60 min. Microsomes were washed twice, with the final wash buffer containing 1 M sodium chloride. Microsomal protein content was assayed by the Lowry method using bovine serum albumin as a standard. The microsomes were stored at −70°.

Assay of Lipid Peroxidation

Unless otherwise stated, the following reaction conditions were used. Reaction mixtures contained 0.6 mg of microsomal protien in a total volume of 0.3 mL of buffer (0.02 M Tris-HCl, 0.15 M KCl, pH 7.4). Mixtures were prepared to contain all the components except the inducer(s) of the lipid peroxidation. Stock solutions of K vitamins and α-tocopherol were in 1% (v/v) Triton X-100 in Tris buffer. The final Triton X-100 concentration in reaction mixtures was 0.04%. Carbon tetrachloride and menadione were dissolved in ethanol. The final ethanol concentration in reaction mixtures was 1.6%. Other components were administered as solutions in Tris buffer. Mixtures were preincubated for 5 min at 37° before the lipid peroxidation reaction was started by the addition of the prooxidant. The Fe^{2+}/ascorbate system was started by the addition of a freshly prepared mixture of ferrosulfate and ascorbate to give final concentrations of 2 and 70 μM, respectively. The NADPH-Fe^{3+}/ATP system was started by the addition of a prewarmed (5 min at 37°) NADPH-generating system to reaction mixtures containing 40 μM ferrichloride and 0.4 mM ATP. The NADPH-CCl_4 system was started by the addition of a prewarmed NADPH-generating system to reaction mixtures containing 5 mM CCl_4. The NADPH-generating system contained in Tris buffer (10 × final concentration) 10 mM NADP, 80 mM glucose-6-phosphate, 25 mM $MgCl_2$, and 10 units of glucose-6-phosphate dehydrogenase. At the end of the incubation, 0.15 mL was used for the assay of TBARS§ [28].

Microsomal Vitamin K Epoxide Reductase

The vitamin K epoxide reductase was assayed as described [26]. Briefly, a reaction mixture (0.1 mL) of microsomal protein (ca. 0.4 mg) and vitamin K epoxide (80 μM) was preincubated for 3 min at 37°, whereafter the reaction was started by the addition of DTT, 5 mM final concentration. After a 10-min incubation period, the reaction was stopped by the addition of 1.4 mL of propanol-2/water (9/5, v/v). The formed vitamin K was extracted with *n*-hexane containing α-tocopherolacetate as internal standard. For the HPLC analysis of vitamin K, a Chromspher C18 column, 100 × 3 mm (Chrompack, Roosendaal, The Netherlands) was used with a mixture of acetonitrile/propanol-2/water (100/8/2, v/v/v) as mobile phase at a flow of 1.2 mL/min. UV detection was at 250 nm. The same system was used for the analysis of vitamin K-hydroquinone. The retention times were (in min) 1.93, 2.94, 4.00, and 4.70 for the hydroquinone, epoxide, internal standard, and vitamin K, respectively.

Chemicals in the purest grade were obtained from commercial suppliers. The epoxide of the K vitamins was prepared by oxidation under alkaline conditions [29]. Cl-K was a gift from Dr. B. Soute (Department of Biochemistry, University of Maastricht).

RESULTS

The reductase, i.e. vitamin K epoxide reductase, of the vitamin K cycle needs a dithiol as reducing cofactor. The physiological dithiol has not been definitively established, although reduced lipoamide was reported as a candidate [30]. Generally, DTT is used as dithiol for *in vitro* vitamin K epoxide reductase activity [21]. Dithiols by themselves are potent inhibitors of microsomal lipid peroxidation, probably by regenerating vitamin E [31]. DTT concentrations that are optimal for *in vitro* vitamin K epoxide reductase (DTT > 2 mM) largely prevented the formation of TBARS induced by either of the lipid peroxidation systems. With 1 mM DTT, microsomal lipid peroxidation was not or only slightly inhibited, principally during the initial 10–20 min of reaction. This DTT concentration was used to test the effect of the vitamin K cycle. Vitamin K (≤50 μM) by itself had no effect on microsomal lipid peroxidation. In the presence of DTT, however, micromolar concentrations of vitamin K almost completely pre-

§ *Abbreviations:* Cl-K, 2-chloro-3-phytyl-1,4-naphthoquinone; DTT, dithiothreitol; TBARS, thiobarbituric acid reactive substances.

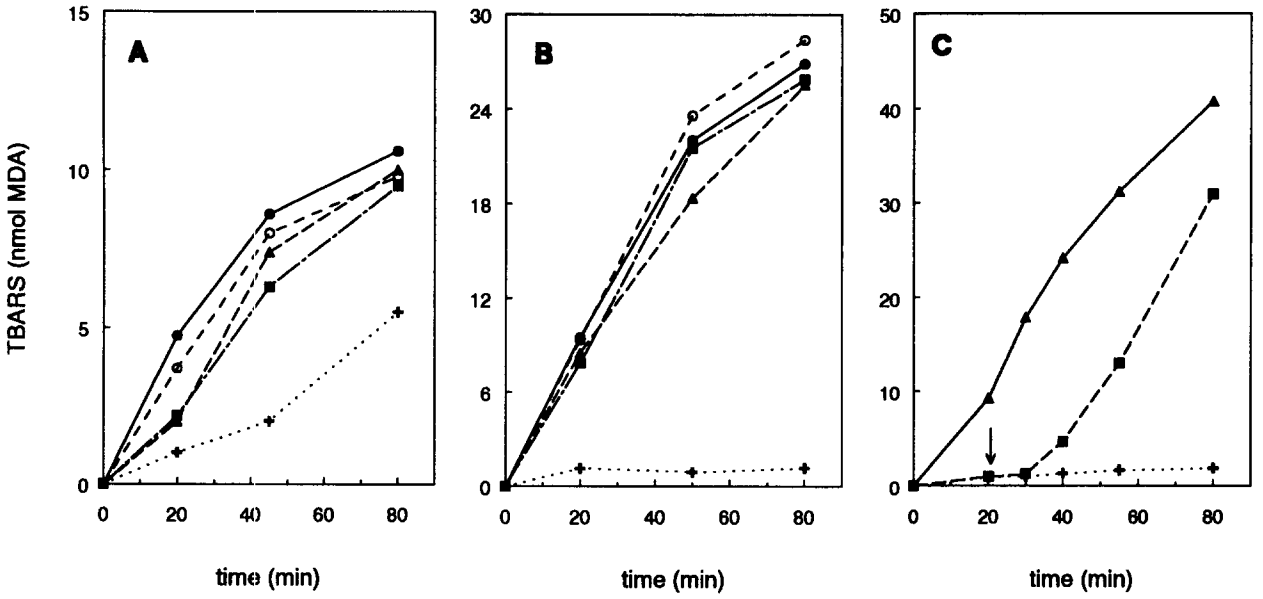


FIG. 1. A, the effect of vitamin K_1 (0.3 μM) plus DTT (1 mM) on Fe^{2+}/ascorbate-induced microsomal lipid peroxidation. Control reaction, ○----○; control plus vitamin K_1, ●----●; control plus DTT, ▲----▲; control plus vitamin K_1 and DTT, +----+; the effect of warfarin (5 μM), ■----■. B, the effect of vitamin K_1 (3 μM) plus DTT (1 mM) on NADPH/CCl_4-induced microsomal lipid peroxidation. For symbols see A. C, the effect of vitamin K_1 (1 μM) plus DTT (1 mM) on NADPH-Fe^{3+}/ATP-induced microsomal lipid peroxidation. At 20 min (arrow), warfarin (5 μM) was added to a vitamin K_1 plus DTT-containing reaction mixture.

vented lipid peroxidation for 80 min. Warfarin, added before or during the reaction, abolished the vitamin K effect (Fig. 1).

The vitamin K effect was concentration-dependent with 90% inhibition at a concentration of 1 μM. Vitamin K_1 (phylloquinone) and vitamin K_2 (menaquinone-4) were equally active, with an IC_{50} value of ca. 0.2 μM (Table 1). Warfarin counteracted the vitamin K effect (Fig. 1, Table 1). The 2-chloro analogue of vitamin K_1 (Cl-K), an inhibitor of the vitamin K-dependent carboxylase [25], also inhibited the antioxidative effect of the vitamin K/DTT combination (Table 1).

TABLE 1. The effect of the vitamin K cycle on lipid peroxidation in rat liver microsomes

	TBARS*
Control	100 ± 11
+DTT (1 mM)	81 ± 6
+K_1 (1 μM)	93.5 ± 8
+DTT (1 mM)	
+K_1 (0.1 μM)	68 ± 19
+K_1 (0.3 μM)	27 ± 12
+K_1 (1 μM)	11.5 ± 4
+K_1 (1 μM) + warfarin (5 μM)	85.5 ± 11
+K_2 (1 μM)	8.5 ± 8
+K_2 (1 μM) + warfarin (5 μM)	92.5 ± 5
+K_2 (3 μM)	5.5 ± 3
+K_2 (3 μM) + Cl-K (50 μM)	85 ± 4.5

Microsomal lipid peroxidation (as TBARS formation) was catalyzed by the NADPH-Fe^{3+}/ATP system for 45 min at 37°.

*TBARS expressed as percent of control reaction. At the test conditions, controls generate ca. 50–80 nmol of MDA equivalents. The data are the mean (±SD) of three to four separate experiments in duplicate.

To establish the role of the hydroquinone of vitamin K, lipid peroxidation was induced after preincubating microsomes in the presence of vitamin K_1 and DTT to allow for the formation of the hydroquinone. The results show declining lipid peroxidation with increasing hydroquinone levels (Table 2). The addition of exogenous dihydroquinone failed to prevent lipid peroxidation, probably due to the rapid oxidation of the hydroquinone before its incorporation into the lipid membrane.

It was observed that a lipid peroxidation reaction could not be stopped by the subsequent addition of vitamin K and DTT. One reason could be the inactivation of vitamin K epoxide reductase during lipid peroxidation. We tested the

TABLE 2. Relationship between vitamin K-hydroquinone and TBARS

Incubation (min)*	Vitamin KH_2 formed†	TBARS
0	0	100
5	0.2	80
10	0.55	52
20	1.3	43
40	2.0	35.5
Control‡	0	84

*A reaction mixture of 0.75 mL of Tris buffer, pH 7.4, containing 5 mg of microsomal protein and 60 μM vitamin K_1 was preincubated for 5 min at 37°, whereafter DTT (5 mM) was added. The incubation was continued. At the time points indicated, 0.1 mL was taken for the analysis of the hydroquinone and 0.04 mL was added to 0.41 mL of a freshly prepared mixture of $FeSO_4$ (2 μM) and ascorbate (70 μM) in buffer. TBARS formation was allowed for 30 min at 37°.

†The amount of vitamin K_1-hydroquinone (KH_2) formed in arbitrary units; peak area ratio KH_2/internal standard (see "Materials and Methods").

‡The control reaction was a 40-min incubation period without vitamin K_1 and DTT. The data are the mean of two independent experiments.

TABLE 3. The effect of lipid peroxidation on vitamin K-epoxide reductase (KOR) activity

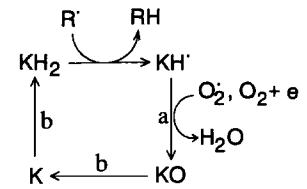
	TBARS (% of control)	KOR (% of control)
+NADPH/CCl_4	100	6.4 ± 4.7
+DTT (1 mM)	81.7 ± 9.3	9.6 ± 1.6
+K_2 (3 μM)	102 ± 8	5.9 ± 0.5
+K_2 + DDT	—	89.4 ± 9.2
+E (5 μM)	20.7 ± 7.5	53.5 ± 10
+E (50 μM)	—	96.7 ± 12.5
+GSH (1 mM)	42.0 ± 0.0	33.8 ± 1.4
+M (1 μM)	—	93.6 ± 6.0

In a total volume of 0.3 mL of Tris-HCl buffer, 0.6 mg of microsomal protein was incubated with or without (= control of KOR) CCl_4 and the NADPH-generating system for 30 min at 37°. An aliquot of 0.15 mL was used for TBARS assay. 0.1 mL of the reaction mixture was diluted once with Tris buffer containing vitamin K_1 epoxide (0.16 mM) and DTT (5 mM). Vitamin K epoxide reduction proceeded for 10 min at 37°. K_2, vitamin K_2; E, α-tocopherol; GSH, reduced glutathione; M, menadione. The data are the mean (±SD) of two separate experiments in triplicate. The appropriate controls were included for each combination. —, no TBARS detectable.

effect of the NADPH/CCl_4 prooxidant system on microsomal vitamin K epoxide reductase. Vitamin K epoxide reductase activity rapidly declined, with 40–50% of the activity being lost within 10 min. The inactivation could be prevented by compounds that inhibit lipid peroxidation such as vitamin K (here vitamin K_2) in combination with DTT, vitamin E (α-tocopherol), reduced glutathione, and menadione (Table 3). Antioxidative enzymes such as superoxide dismutase and catalase did not prevent the inactivation of the reductase (data not shown).

DISCUSSION

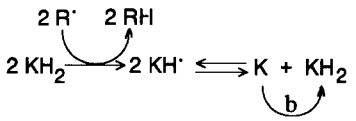
The results presented in the study reveal the potent inhibitory activity of the vitamin K cycle in microsomal lipid peroxidation. The potential of vitamin K as an antioxidant has been investigated by several groups. One of the earliest studies showed a stimulation of microsomal lipid peroxidation by vitamin K in contrast to the effect of menadione [16]. Other groups reported that vitamin K inhibited lipid peroxidation. Relatively high concentrations (IC_{50} values far above 0.1 mM) were needed, however, to observe the effect [17–19]. In our experiments, no antioxidant effect of vitamin K (concentrations up to 50 μM) by itself was observed. This is also true for vitamin K epoxide (data not shown). Menadione is a potent inhibitor of NADPH-dependent microsomal lipid peroxidation (see also Table 3). Diversion of the electrons of NADPH-cytochrome P450 reductase from the membranous compartment is believed to underlie the mechanism of menadione inhibition [12, 16]. The K vitamins with a related naphtoquinone structure are highly fat soluble and therefore will not be available (unlike menadione) as one-electron acceptors of the NADPH-cytochrome P450 reductase-derived electrons at the membrane/water interphase. The reduced form of vitamin K (vitamin K-hydroquinone), on the other hand, has strong antioxidant activity, being 10–100-fold more potent than α-tocopherol and ubiquinol as a radical scavenger when tested in solution [14, 20].



SCHEME 1 — Proposed reaction routes for the antioxidant action of vitamin K. KH_2, KH*, KO, and K represent the hydroquinone, semiquinone, epoxide, and quinone form of vitamin K, respectively. R* is a radical species. The complete cycle is involved, where a = γ-glutamylcarboxylase and b = vitamin K epoxide reductase.

In the vitamin K cycle, vitamin K-hydroquinone is oxidized to vitamin K epoxide, which in turn is reduced to vitamin K and subsequently to vitamin K-hydroquinone. The epoxidation, mediated by the vitamin K-dependent γ-glutamylcarboxylase, provides the energy for the abstraction of the γ-hydrogen of protein-bound glutamates [21]. Epoxidation and carboxylation need not be coupled per se [26]. The involvement of the vitamin K cycle in the vitamin K/DTT-dependent suppression of microsomal lipid peroxidation is emphasized by the observed warfarin (Fig. 1, Table 1) and Cl-K (Table 1) inhibition. Warfarin inhibits vitamin K epoxide reductase, which mediates the reduction steps in the cycle [21], whereas Cl-K is a potent inhibitor of the carboxylase/epoxidase reaction [25]. The results implicitly demonstrate that the radical-scavenging vitamin K species is continuously regenerated.

From the foregoing arguments, one can reasonably assume that the antioxidant species of the vitamin K cycle is vitamin K-hydroquinone, which is formed out of the quinone by the DTT-dependent vitamin K epoxide reductase. Additional proof is provided by the data showing an inverse relationship between vitamin K_1-hydroquinone and TBARS (Table 2). The antioxidant effect is probably due to radical chain breaking. Alternatively, the action may be the result of the regeneration of active vitamin E from the tocopheroxyl radical [14]. In any event, the reaction product of vitamin K-hydroquinone and the free radical, which in all probability is the semiquinone radical, is processed in the vitamin K cycle to regenerate the hydroquinone. A radical-based mechanism for the γ-glutamylcarboxylase-mediated γ-glutamyl hydrogen abstraction has been postulated with a central role for the semiquinone of vitamin K [23–25]. The semiquinone was thought to react with superoxide anion [24] or with oxygen [25], thereby forming a reactive intermediate that breaks the γ-glutamyl hydrogen bound concomitantly with epoxidation. According to this mechanism, the vitamin K cycle intrinsically functions as radical scavenger, thus accounting for our results (Scheme 1). Recent insights into vitamin K epoxidation reject a role for the radical mechanism, inclining instead toward a base-catalyzed mechanism via the dioxetane anion for the abstraction of the γ-glutamyl hydrogen [22]. There-



SCHEME 2 — The route for the involvement of vitamin K epoxide reductase alone (for symbols see Scheme 1). See text for further explanation.

fore, an alternative mechanism for the antioxidant effect could be as presented (Scheme 2); in common with (benzo)quinones [32], there may exist a disproportionation equilibrium between the semiquinone, the hydroquione, and the quinone forms. The vitamin K epoxide-mediated reduction of the quinone shifts the equilibrium to the right (Scheme 2). This scheme does not explain, however, the effect of Cl-K.

The vitamin K cycle is embedded in the interior of the rough endoplasmatic reticulum [21]. This indicates that the reactive radical species generated from the NADPH-Fe^{3+}/ATP, the Fe^{2+}/ascorbate, or the NADPH/CCl_4 system penetrate into the aprotic interior of the membrane where they initiate lipid peroxidation. Koga and Nakano [33], in studying the effect of various antioxidants in iron-induced microsomal lipid peroxidation, also concluded that only intramembranous O_2^- leads to lipid peroxidation. The inability of SOD and catalase to inhibit microsomal lipid peroxidation [34] or to protect vitamin K epoxide reductase from inactivation (our own observation) is compatible with this. The sensitivity of microsomal vitamin K epoxide reductase for lipid peroxidation is understandable given its absolute requirement of a membranous environment [30, 35]. Furthermore, the catalytic active thiol groups of the reductase may be extra vulnerable to oxygen-reactive species.

Vitamin E is recognized as the principal fat-soluble antioxidant protecting cellular membranes against oxidative attack, with approximately 1 molecule of vitamin E present per 1000–2000 phospholipid molecules [4, 6]. Moreover, cells are equipped with biosystems to regenerate vitamin E [6]. The vitamin K cycle also effectively regenerates the active cofactor, with 1 molecule of vitamin K being used 1000–10,000 times in the carboxylase reaction [15]. Furthermore, the capacity of the vitamin K cycle can be high [27], and some organs specifically accumulate vitamin K [36, 37]. The concentration of vitamin K for the suppression of microsomal lipid peroxidation (micromolar range) is, however, far beyond the physiological tissue concentrations, which are in the range of pmoles/gram of tissue. Due to compartmentalization, however, the vitamin K cycle could act as an antioxidant at specific intracellular sites, for instance, to protect the vitamin K cycle itself from oxidative damage. The protective effect of vitamin K in the early phase of CCl_4-induced toxicity in rat hepatocytes may have resulted from the antioxidative action [38]. An *in vivo* function for vitamin K as antioxidant will have to be established in future experiments. Thus far, no adverse effects other than risks of bleeding are known for lifelong warfarin treatment. One reason could be that anticoagulant therapy does not contain complete inhibition of the vitamin K cycle [27].

In summary, the vitamin K cycle intrinsically bears potential antioxidative properties. Its intracellular compartmentalization could contain a localized defense system against free radicals. Dietary intake of vitamin K may not only be essential for the maintenance of heamostasis but may, together with other phytonutrients, strengthen cellular defense against oxidative stress.

References

1. Slater TF, Free radical mechanism in tissue injury. *Biochem J* **222:** 1–15, 1984.
2. Halliwell B and Gutteridge JMC, Lipid peroxidation: a radical chain reaction. In: *Free Radicals in Biology and Medicine* (Eds. Halliwell, B, and Gutteridge, JMC), pp. 188–266. Clarendon Press, Oxford, 1989.
3. Rice-Evans C and Burdon R, Free radical-lipid interactions and their pathological consequences. *Prog Lipid Res* **32:** 71–110, 1993.
4. Burton GW and Ingold KU, Vitamin E as an *in vitro* and *in vivo* antioxidant. *Ann NY Acad Sci* **570:** 7–23, 1989.
5. Packer L, Maguire JJ, Mehlhorn RJ, Serbinova E and Kagan VE, Mitochondria and microsomal membranes have a free radical reductase activity that prevents chromanoxyl radical accumulation. *Biochem Biophys Res Commun* **159:** 229–235, 1989.
6. Packer L and Kagan VE, Vitamin E: the antioxidant harvesting center of membranes and lipoproteins. In: *Vitamin E in Health and Disease*. Eds. Packer L and Fuchs J, pp. 179–192. Marcel Dekker Inc, New York, 1993.
7. Sies H, Stahl W and Sundquist AR, Antioxidant functions of vitamins. *Ann NY Acad Sci* **669:** 7–20, 1992.
8. Sanz MJ, Fernandiz ML, Cejudo M *et al.*, Influence of a series of natural flavonoids on free radical generating systems and oxidative stress. *Xenobiotica* **24:** 689–699, 1994.
9. Frankel EN, Kanner J, German JB, Parks E and Kinsella JE, Inhibition of oxidation of human low-density lipoprotein by phenolic substances in red wine. *Lancet* **341:** 454–457, 1993.
10. Frei B, Kim MC and Ames BN, Ubiquinol-10 is an effective lipid-soluble antioxidant at physiological concentrations. *Proc Natl Acad Sci USA* **87:** 4879–4883, 1990.
11. Fernandez-Villamil S, Dubin M, Galeffi C and Stoppani AOM, Effects of mansenones on lipid peroxidation, P450 monooxygenase activity, and superoxide anion regeneration by rat liver microsomes. *Biochem Pharmacol* **40:** 2343–2351, 1990.
12. Dubin M, Fernandez-Villamil SH and Stoppani OM, Inhibition of microsomal lipid peroxidation and cytochrome P-450-catalysed reactions by β-lapachone and related naphtoquinones. *Biochem Pharmacol* **39:** 11151–1160, 1990.
13. Kagan V, Serbinova E and Packer L, Antioxidant effects of ubiquinones in microsomes and mitochondria are mediated by tocopherol recycling. *Biochem Biophys Res Commun* **169:** 851–857, 1990.
14. Mukai K, Itoh S and Morimoto H, Stopped-flow kinetic study of vitamin E regeneration with biological hydroquinones (reduced forms of ubiquinone, vitamin K, and tocopherol quinone) in solution. *J Biol Chem* **267:** 22277–22281, 1992.
15. Vermeer C, γ-Carboxylglutamate-containing proteins and the vitamin K-dependent carboxylase. *Biochem J* **266:** 625–636, 1990.
16. Wills ED, Effects of vitamin K and naphtoquinones on lipid

peroxide formation and oxidative demethylation by liver microsomes. *Biochem Pharmacol* **21:** 1879–1883, 1972.
17. Canfield LM, Davy LA and Thomas GL, Anti-oxidant/pro-oxidant reactions of vitamin K. *Biochem Biophys Res Commun* **128:** 211–219, 1985.
18. Talcott RE, Smith MT and Gianni DD, Inhibition of microsomal lipid peroxidation by naphtoquinones: structure-activity relationships and possible mechanisms of action. *Arch Biochem Biophys* **241:** 88–94, 1985.
19. Ohyashiki T, Yabunaka Y and Matsui K, Antioxidant effects of vitamin K homologues on ascorbic acid/Fe^{2+}-induced lipid peroxidation of lecithin liposomes. *Chem Pharm Bull* **39:** 976–979, 1991.
20. Mukai K, Morimoto H, Kikuchi S and Nagaoka S, Kinetic study of free-radical-scavenging action of biological hydroquinones (reduced forms of ubiquinone, vitamin K and tocopherol quinone) in solution. *Biochim Biophys Acta* **1157:** 313–317, 1993.
21. Suttie JW, Recent advances in hepatic vitamin K metabolism and function. *Hepatology* **7:** 367–376, 1987.
22. Dowd P, Hershline R, Ham S-W and Naganathan S, Vitamin K and energy transduction: a base strength amplification mechanism. *Science* **269:** 1684–1691, 1995.
23. Johnson BC, Giardot J-M, Suen ET, Mack DO, Floyd RA and Delaney R, Biological role of vitamin K at the molecular level. *Wld Rev Nutr Diet* **31:** 202–209, 1978.
24. Canfield LM and Ramelow U, Vitamin K-dependent carboxylase: evidence for a semiquinone radical intermediate. *Arch Biochem Biophys* **230:** 389–399, 1984.
25. Chueng AY, Wood GM, Funakawa S, Grossman CP and Suttie JW, Vitamin K-dependent carboxylase: substrates, products, and inhibitors. In: *Current Advances in Vitamin K Research*. Ed. Suttie JW, pp. 3–16. Elsevier, New York, 1988.
26. Mosterd JJ and Thijssen HHW, The long-term effects of the rodenticide, brodifacoum, on blood coagulation and vitamin K metabolism in rats. *Br J Pharmacol* **104:** 531–535, 1991.
27. Mosterd JJ and Thijssen HHW, The relationship between the vitamin K cycle inhibition and the plasma anticoagulant response at steady-state S-warfarin conditions in the rat. *J Pharmacol Exp Ther* **260:** 1081–1085, 1992.
28. Cecchini R, Aruoma OI and Halliwell B, The action of hydrogen peroxide on the formation of thiobarbituric acid-reactive material from microsomes, liposomes or from DNA by bleomycin or phenantroline: artefacts in the thiobarbituric acid test. *Free Rad Res Commun* **10:** 245–258, 1990.
29. Tishler M, Fieser LF and Wendler NL, Hydro, oxido and other derivatives of vitamin K_1 and related compounds. *J Am Chem Soc* **62:** 2866–2871, 1940.
30. Thijssen HHW, Janssen PG and Vervoort LTM, Microsomal lipoamide reductase provides vitamin K epoxide reductase with reducing equivalents. *Biochem J* **297:** 277–280, 1994.
31. Murphy ME, Scholich H, Wefers H and Sies H, Alpha-tocopherol in microsomal lipid peroxidation. *Ann NY Acad Sci* **570:** 480–486, 1989.
32. Wardman P, Bioreductive activation of quinones: redox properties and thiol reactivity. *Free Rad Res Commun* **8:** 219–229, 1990.
33. Koga S and Nakano M, A high involvement of O_2^- possibly generated in inner membranes for iron-induced microsomal lipid peroxidation. *Biochem Biophys Res Commun* **186:** 1087–1093, 1992.
34. Ischiropoulos H, Kumae T and Kikkawa Y, Effect of interferon-inducers on superoxide anion generation from rat liver microsomes detected by lucigenin chemiluminescence. *Biochem Biophys Res Commun* **161:** 1042–1048, 1989.
35. Hildebrandt EF, Preusch PC, Patterson JL and Suttie JW, Solubilization and characterization of vitamin K epoxide reductase from normal and warfarin-resistant rat liver microsomes. *Arch Biochem Biophys* **228:** 480–492, 1984.
36. Thijssen HHW and Drittij-Reijnders MJ, Vitamin K distribution in rat tissue: dietary phylloquinone is a source of tissue menaquinone-4. *Br J Nutr* **72:** 415–425, 1994.
37. Thijssen HHW and Drittij-Reijnders MJ, Vitamin K status in human tissues: tissue-specific accumulation of phylloquinone and menaquinone-4. *Br J Nutr* **75:** 121–127, 1996.
38. Itoh Y and Terano A, Effect of vitamin K on carbon tetrachloride-induced cell damage in primary cultured rat hepatocytes. *Gastroenterology* **25:** 463–470, 1990.