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ANTI-OXIDANT/PRO-OXIDANT REACTIONS OF VITAMIN K

Louise M. Canfield*, Laura A. Davy, and Gregory L. Thomas

Departments of Biochemistry & Biophysics and Animal Science
Texas Agricultural Experiment Station
Texas A&M University
College Station, Texas 77843-2471

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SUMMARY: **Experiments were designed to measure O_2 consumption caused by the oxidation of linoleic acid. These experiments show that vitamin K has antioxidant activity and that the reduction in linoleic acid oxidation is directly dependent upon vitamin K concentration. Conversely, vitamin K hydroquinone enhances linoleic acid oxidation in the absence of iron catalyst, again in a concentration dependent manner. At equimolar concentrations vitamin K is about 80% as effective as vitamin E as an antioxidant. Vitamin E inhibits the oxidation of linoleic acid catalyzed by vitamin K hydroquinone. Vitamin E also strongly inhibits vitamin K dependent formation of both vitamin K epoxide and gamma-carboxyglutamic acid (gla). The significance of these observations to vitamin K action *in vivo* is discussed.**

INTRODUCTION: A variety of biological systems require the reaction of vitamin K with oxygen (1-4); however, the chemistry and thus the enzymology of these reactions is poorly understood. The enzymatic reaction of vitamin K which has received the most attention is vitamin K-dependent carboxylation. In this reaction, glutamic acid residues in prothrombin and other proteins of the blood coagulation cascade are carboxylated to form gamma-carboxyglutamic acid concomitant with the incorporation of oxygen into vitamin K hydroquinone to produce vitamin K epoxide (5). Vitamin K carboxylase is membrane-bound, and lipid requirements for carboxylation have been reported (4,6). Thus, understanding of the reactions of vitamin K and oxygen in the presence of lipids is fundamental to our knowledge of vitamin K function. Surprisingly

*To whom inquiries should be addressed at the Department of Animal Science, Texas A&M University, College Station, Texas 77843.

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little is known about these reactions. Further, recent reports of toxic effects of vitamin K on cell membranes (7-9) and vitamin E antagonism of vitamin K action (10) provide additional evidence that a thorough investigation of the reactions of vitamin K and oxygen with biological lipids is needed. Thus, the present report describes a preliminary study of the effects of vitamin K or vitamin K hydroquinone on the oxidation of linoleic acid.

MATERIALS AND METHODS:

ANIMALS: Male Sprague-Dawley rats (200-250 g) were maintained 3-5 days on laboratory chow. Eighteen hours prior to sacrifice, they were injected intraperitoneally with sodium Warfarin (5.0 mg/kg body wt.) to induce activity of vitamin K-dependent carboxylase (epoxidase).

PREPARATION OF VITAMIN K CARBOXYLASE (EPOXIDASE): Semi-purified preparations of Vitamin K carboxylase (Complex A) were prepared by a modification of the method previously reported (6,12). Final preparations were stored in 0.33 M K_2HPO_4, 0.20% Renex 30, 10.0% glycerol, 1.0 mM DTT (dithiothreitol), pH 7.2 at -70°C until use.

ASSAYS:

Oxidation of Linoleic Acid: All buffers and solvents were saturated with argon prior to use. Reaction mixtures contained 20.0 mM linoleic acid emulsified in 0.10 M sodium phosphate, 0.02 mM EDTA (ethylenediaminetetraacetic acid) pH 6.2, (13) and, for testing of antioxidant activity, either vitamin E or vitamin K in $(Me)_2SO$ (dimethylsufoxide). Reaction mixtures were equilibrated at 25°C in a sealed cuvette at known oxygen concentrations. Oxidation was initiated by injecting from a gas-tight syringe either a freshly prepared aqueous solution of $Fe(NH_4)_2(SO_4)_2$, or argon-saturated ethanolic solutions of vitamin K hydroquinone. Oxygen consumption in the continuously stirred solutions was monitored using a YSI Model 53 oxygen meter equipped with a Clark electrode and a Houston Instruments strip chart recorder modified with a voltage divider circuit to provide 5X amplification of the signal.

Vitamin K Carboxylase (Epoxidase):

A) Detailed methodology for quantitation of vitamin products has been described elsewhere (11). Briefly, standard reaction mixtures, in a total volume of 0.50 ml, contained 0.50 to 1.00 mg carboxylase protein preparation and 0.50 mM peptide substrate in 0.10% Renex 30, 0.33 M K_2HPO_4, pH 7.2. Reactions were initiated by the addition of vitamin K hydroquinone from argon-saturated ethanolic solutions to give a final concentration in the assay of 0.04 mM. Reaction mixtures were incubated for 20 minutes at 25°C, and extracted with chloroform:methanol (2:1 v/v). The chloroform layers containing the extracted vitamins were evaporated to dryness under nitrogen and the recovered vitamins dissolved in ethanol for injection onto the HPLC (11).

B) Preparations of vitamin K-dependent carboxylase (0.50 to 1.0 mg protein ml^{-1}) were added to 0.10% Renex 30, 0.33M K_2HPO_4, pH 7.2. and peptide substrate added to a final concentration of 0.50 mM. Reactions were initiated by the stepwise addition of

0.01 mCi $NaH^{14}CO_3$ and sufficient vitamin K hydroquinone to give a final assay concentration of 0.04 mM. Reaction mixtures (0.50 ml) were incubated 20 minutes at 25°C., quenched by the addition of TCA and assayed for incorporation of $^{14}CO_2$ into peptide as previously described (12).

HPLC ANALYSES: HPLC analyses were performed as previously described (11) on a Waters HPLC (Model 6000A) equipped with a solvent programmer (Model 660), an auxiliary pump (Model M45) and variable wavelength detector (Model 450). For analytical chromatography, samples (10-50 µl) were injected onto a Nova-Pak Radial Pak-L-Liquid Chromatography Cartridge, eluted with a convex gradient (curve #3) from ethanol:H_2O (90:10 v/v) to ethanol: hexane (90:10 v/v) at 1.5 ml min^{-1} and monitored at 254 nm.

PREPARATION AND PURIFICATION OF THE VITAMINS: Vitamin K hydroquinone and vitamin K epoxide were synthesized as previously described by sodium hydrosulfite reduction and hydrogen peroxide oxidation, respectively, of the purified quinone (12). Vitamin K, vitamin K hydroquinone, and vitamin K epoxide were quantitated from their absorbances in the ultraviolet using extinction coefficients of 18,900 M^{-1} cm^{-1} at 248 nm for the quinone, 44,100 M^{-1} cm^{-1} at 245 nm for the hydroquinone, and 22,110 M^{-1} cm^{-1} for the epoxide (11), using a Beckman DU-7 spectrophotometer.

CHEMICALS: Renex 30 was obtained from ICI Chemicals (Wilmington, Delaware). Warfarin, vitamin K (2-methyl-3-phytyl-1, 4-napthoquinone) and peptide substrate (phe-leu-glu-glu-ile), linoleic acid, and d,l-α-tocopherol (vitamin E) were from Sigma Chemical Co. (St. Louis, Mo.). Ferrous ammonium sulfate [$Fe(NH_4)_2(SO_4)_2$] was from MCB (E. Merck, Darmstadt, Germany), $NaH^{14}CO_3$ (58 mCi/mMol) was from ICN Chemical & Radioisotope Division (Irvine, California). All solvents were HPLC grade from Burdick and Jackson Laboratories (Muskegon, Michigan).

RESULTS AND DISCUSSION: Oxidation of linoleic acid was measured by following the initial rates of oxygen uptake as previously described (14). The effects of vitamin K, vitamin K hydroquinone and vitamin E on linoleic acid oxidation are compared in Table 1. Addition of 1.0 mM vitamin K inhibited the initial rate of oxygen consumption by 57%. The rate of oxygen consumption was inversely proportional to vitamin K concentrations. For comparison, vitamin E in the same concentration inhibited linoleic acid oxidation by 73%. Thus vitamin K was only slightly less effective (~80%) an antioxidant than was vitamin E in this system. No attempt was made to isolate the oxidation product(s). In view of the structural similarity of vitamin K to vitamins A and E, both of which are widely used antioxidants (15), our results are not surprising, although to our knowledge, antioxidant reactions of vitamin K *in vitro* have not previously been reported. However, protective effects of vitamin K against oxygen

TABLE I
EFFECTS OF VITAMIN K AND VITAMIN K HYDROQUINONE
ON LINOLEIC ACID OXIDATION

	ADDITION	Initial Rate nmols O_2/Min.
$+Fe^{++}$	None	300
	Vitamin K, 1.0 mM	130
	2.0 mM	60[1]
	Vitamin E, 1.0 mM	80
$-Fe^{++}$	Vitamin K hydroquinone:	
	0.50 mM	440[1]
	0.10 mM	150
	0.05 mM	90[1]
	Vitamin K hydroquinone, 0.1 mM + Vitamin E, 0.1 mM	60

Reaction mixtures (2.0 ml) containing 20.0 mM linoleic acid, 0.10 M Na_2HPO_4, 0.02 mM Na_2EDTA, and the antioxidant of choice were equilibrated in an oxygen meter at 25° C. Reactions were initiated by the addition of $Fe(NH_4)_2SO_4$ to provide a final concentration in the reaction mixture of 0.020 mM, or by addition of vitamin K hydroquinone as described in Methods.

[1]Single experiments. All other numbers are averages of duplicate measurements obtained in three separate experiments.

toxicity *in vivo* have been observed. Dietary supplements of vitamin K or menadione (vitamin K_3) increased survival of mice following exposure to oxygen, (16) and menadione protected against pulmonary symptoms of oxygen poisoning (9).

In contrast, vitamin K hydroquinone stimulated oxidation of linoleic acid (Table 1). In these experiments, the hydroquinone was added to equilibrated suspensions of linoleic acid containing no iron-EDTA catalyst. As shown in Fig. 1, oxygen consumption was detected immediately upon the addition of vitamin K hydroquinone. The rate of oxygen uptake was nearly linear and continued until all oxygen in the cuvette was depleted (approximately 15 minutes). The rate of oxygen

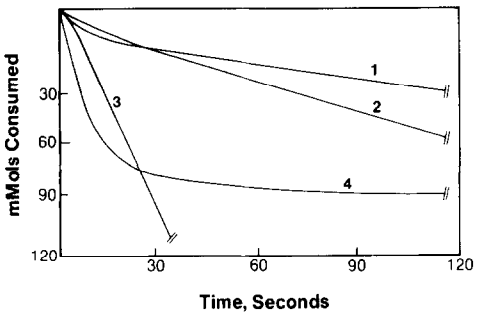

Figure 1 Oxidation of linoleic acid by vitamin K hydroquinone. Oxygen uptake by 0.10 mM vitamin hydroquinone in 0.10 M Na_2HPO_4, pH 6.2 was measured in the presence of :

(1) no addition
(2) 20.0 mM linoleic acid and 1 mM vitamin E
(3) 20.0 mM linoleic acid
(4) Oxidation of 20.0 mM linoleic acid by 0.02 mM $Fe(NH_4)_2SO_4$ in the absence of vitamin K hydroquinone is shown for comparison.

All reactions were conducted in an oxygen meter at 25°C. in a final volume of 2.0 ml as described in methods.

consumption increased with hydroquinone concentration (Table 1). The amount of oxygen consumed by 20.0 mM linoleic acid in the presence of 0.10 mM vitamin K hydroquinone by 15 minutes was 4-fold greater than that consumed in the iron-catalyzed control (data not presented). Under otherwise identical reaction conditions, oxygen consumption by vitamin K hydroquinone in the absence of linoleic acid was minimal (Fig.1). When completed reaction mixtures containing vitamin K hydroquinone and linoleic acid were immediately extracted and analyzed on HPLC, the only form of the vitamin detected was oxidized vitamin K. Thus the species which potentiates lipid oxidation is apparently the semiquinone formed on oxidation of vitamin K hydroquinone (17).

Although toxic effects of vitamin K are relatively rare, some have been reported, particularly as a result of topical application (18). Furthermore, vitamin K has been implicated in metastasis in experimental tumor models (19-22). Thus, to the extent that carcinogenesis is potentiated by free radicals, formation of vitamin K semiquinone from oxidation of the hydroquinone or reduction of the quinone in membranes could be biologically significant. In fact, it has recently been proposed that one function of DT

TABLE 2
INHIBITION OF VITAMIN K-DEPENDENT CARBOXYLASE AND EPOXIDE FORMATION BY VITAMIN E

ADDITIONS	CARBOXYLATION dpm/mg Protein	CARBOXYLATION % Control	EPOXIDE FORMATION nMols/ml	EPOXIDE FORMATION % Control
None	31,000	100	10.0	100
+ Vitamin E				
0.040 mM	20,000	66	6.0	60
1.00 mM	0	0	0	0

Solutions of vitamin E in $(Me)_2SO$ were added to enzyme, peptide and phosphate buffer. Reactions were initiated by addition of vitamin K hydroquinone (and $Na^{14}HCO_3$ for carboxylase assays) as described in Methods. Numbers are the averages of measurements obtained in duplicate experiments.

diaphorase in microsomes is to limit semiquinone formation by reducing quinones to hydroquinones (23). Addition of 1.0 mM vitamin E to mixtures of 0.10 mM vitamin K hydroquinone and 20.0 mM linoleic acid significantly inhibited oxidation of linoleic acid by vitamin K hydroquinone (Fig. 1), apparently by scavenging of vitamin K semiquinone (10,15).

Inhibition of prothrombin synthesis (24,25) and a decrease in vitamin-K dependent coagulation factors occur in response to high doses of vitamin E (26). In preliminary reports (27,28) inhibition of vitamin K-dependent carboxylation by vitamin E was suggested as an explanation for these lesions, however, relatively high (10 mM) concentrations were required to produce significant inhibition. Thus in view of these reports and the inhibition of hydroquinone-catalyzed linoleic acid oxidation described above, we investigated the effect of vitamin E on vitamin K-dependent carboxylation and epoxide formation. As shown in Table 2, neither carboxylation nor epoxide formation were detected in the presence of 1.0 mM vitamin E. At vitamin E concentrations equimolar with vitamin K hydroquinone, approximately 40% inhibition of both carboxylation and epoxide formation was observed. In comparison, (Table 1) hydroquinone-catalyzed linoleic acid oxidation was inhibited 60% by equimolar

concentrations of vitamin E. Inhibition by vitamin E in the two systems is remarkably similar given the complexity of the two reaction mixtures and lability of the reaction components. As stated above, both linoleic acid and vitamin K hydroquinone were relatively stable to oxidation prior to mixing.

We have recently shown (12) that vitamin K semiquinone is required for vitamin K carboxylation and epoxide formation. A number of radical species could be present in our reaction mixtures with which vitamin E might react. However based on the results presented here, inhibition of vitamin K-dependent carboxylation by scavenging of vitamin K semiquinone is a likely explanation of vitamin E antagonism of vitamin K action (24-26).

In summary, vitamin K inhibits linoleic acid oxidation. At 1.0 mM concentrations, vitamin K was about 80% as effective as vitamin E in decreasing oxygen consumption by linoleic acid. In contrast, vitamin K hydroquinone potentiates the oxidation of linoleic acid. This effect may be related to recent reports of vitamin K toxicity. Oxidation of hydroquinone-catalyzed oxidation of linoleic acid is reversed by vitamin E. Vitamin E also inhibits both vitamin K-dependent carboxylation and epoxide formation. It is proposed that vitamin E inhibits vitamin K-dependent carboxylation and epoxide formation as well as vitamin K hydroquinone-catalyzed linoleic acid oxidation by scavenging vitamin K semiquinone. Scavenging of vitamin K semiquinone by vitamin E could explain impaired blood coagulation processes resulting from excess dosages of vitamin E (24-28).

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