INEFFECTIVENESS OF PHYLLOQUINONE EPOXIDE AS AN INHIBITOR OF PROTHROMBIN SYNTHESIS IN THE RAT

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Received September 1,1974

SUMMARY

Phylloquinone epoxide (vitamin K_1-oxide), a metabolite of phylloquinone, does not inhibit prothrombin synthesis when administered in high doses to Sprague-Dawley and warfarin-resistant rats. Further, it does not accumulate to presumed inhibitory levels in the livers of rats given physiological doses of ^{3}H-phylloquinone when they are anticoagulated with warfarin. These data do not support the Bell-Matschiner hypothesis that warfarin exerts its action by inhibiting the vitamin K oxide reductase which results in the accumulation of vitamin K oxide and the inhibition of vitamin K at its active site. Rather, our data support the view that vitamin K and warfarin combine at different sites with a single regulatory protein which serves as a conformational switch for prothrombin synthesis.

INTRODUCTION

Phylloquinone epoxide (vitamin K_1 oxide) was isolated from livers of rats anticoagulated with warfarin (3-α-phenyl-β-acetylethyl-4-hydroxycoumarin) by Matschiner et al. (1) and later identified as a normal metabolite of vitamin K in animals. These workers (2) then reported that phylloquinone oxide had the same biological activity as phylloquinone in the vitamin K deficient rat but was much less active in the warfarin anticoagulated rat suggesting that warfarin inhibited the reductase required for the normal conversion of phylloquinone oxide to phylloquinone.

Bell and Matschiner (3) then attempted to document the view that vitamin K oxide was not only a metabolite of vitamin K but,

in fact, a competitive inhibitor of the vitamin at its active site. Various combinations of vitamin K and vitamin K oxide were administered to warfarin anticoagulated rats and the prothrombin values observed over a period of several hours. There appeared to be a variable effect of vitamin K oxide in the presence of vitamin K and warfarin to reduce the prothrombin output. Subsequently, in experiments employing ^{3}H-labeled vitamin K_1 and vitamin K_1 oxide, the vitamin K oxide/vitamin K_1 ratios were found to be elevated in animals receiving warfarin as opposed to controls, but the hepatic vitamin K oxide/vitamin K ratios obtained in rats pretreated with warfarin receiving various doses of vitamin K from 5 to 100 μg varied over too small a range (0.9 to 1.9) to convincingly account for the changes in prothrombin response (0-60%) (4). Finally, in warfarin resistant rats where vitamin K_1 oxide appeared to accumulate in the absence of warfarin, "inhibitory" ratios of vitamin K oxide to vitamin K_1 were obtained in the absence of warfarin at 100% plasma prothrombin levels (5). Since the Bell-Matschiner hypothesis has been seriously tested only in animals receiving warfarin, which drug has been postulated by others (6,7) to combine with the receptor protein for vitamin K_1, the putative anticoagulant effect of vitamin K oxide has remained in doubt.

In this communication, we report the results of two experiments designed to test the Bell-Matschiner hypothesis. In the first, physiological amounts of vitamin K were administered to rats receiving the minimum effective dose of warfarin and the hepatic vitamin K_1 oxide/vitamin K ratios observed. In the second, vitamin K oxide was administered in high doses to normal and warfarin resistant rats and prothrombin levels observed. Neither experiment leads us to believe that vitamin K_1 oxide

is an inhibitor of vitamin K_1 in regulating prothrombin synthesis. A preliminary report of this work has been made (8).

MATERIALS AND METHODS

Normal male rats of the Sprague-Dawley strain and homozygous warfarin resistant males crossbred from the wild mutant Rattius novvegicas (9) with Sprague-Dawley rats, both weighing approximately 250 g were fed Purina rat chow supplemented with low levels of vitamin K. Only animals with initial prothrombin levels of 80-110% of normal, as determined with the one stage method of Hjort (10), were used.

^{3}H-Phylloquinone labeled in the 5 position with a specific activity of 17 Ci/mmole (gift of Hoffmann-La Roche, Basel) was administered to rats and also used as a source of ^{3}H-phylloquinone epoxide (7). Phylloquinone-5-^{3}H in an amount of 3 µg was dissolved in 1 ml of absolute ethanol in a 3 ml reaction flask. 1 mg of anhydrous Na_2CO_3 was added, and the flask heated in a mineral oil bath to 80.0°C ± 0.2°C. The mixture was stirred constantly and 0.1 ml of a 30% solution of H_2O_2 was added over a 15 minute period. The reaction was continued for a period of 30 minutes, diluted to 10 ml with H_2O, and extracted with diethyl ether. The vitamin K_1 oxide was purified to constant specific activity by reverse and direct phase TLC. The yield of purified vitamin K oxide was 74%. Both the ^{3}H-phylloquinone and the ^{3}H-phylloquinone epoxide were solubilized in 2% Emulphor 620 in physiologic saline for infusion into Sprague-Dawley and warfarin resistant rats.

The hepatic vitamin K_1 oxide/vitamin K ratios were determined by extraction of the livers with chloroform methanol 2:1 (4,11) and the lipid extract chromatographed on thin-layer plates with benzene in which system vitamin K and vitamin K oxide

run together at an R_f of 0.40. The radioactive band was then scraped, extracted with ether and rechromatographed on TLC plates impregnated with 5% paraffin in petroleum ether. These reverse phase plates were developed with acetone: H_2O 92:8 in which system vitamin K and vitamin K oxide move at R_f values respectively 0.39 and 0.60. These reverse phase plates were again scanned and the ^{3}H-vitamin K and ^{3}H-vitamin K oxide bands counted.

RESULTS AND DISCUSSION

In order to avoid the pharmacologic effect of high doses of vitamin K upon vitamin K_1 oxide formation in the presence of warfarin, we sought to employ a dose of vitamin K approximating the minimum daily requirement of the vitamin which is about 1 µg per rat. We infused 50 nanograms of ^{3}H-vitamin K_1 per hour into Sprague-Dawley rats, half of which had received a minimum effective dose of warfarin intraperitoneally (1 mg/kg body weight) one half hour prior to infusion. The results obtained are presented in Table I. The animals which received this low level of vitamin K without warfarin had hepatic vitamin K oxide/vitamin K ratios of 0.04 ± 0.01 at both 2 and 4 hours after infusion in agreement with values obtained by Matschiner *et al.* (1) when traces of ^{3}H-vitamin K was administered by mouth. Prothrombin levels were 100% of control at both 2 and 4 hours. In the warfarin treated rats, the prothrombin levels declined at the expected turnover rate of prothrombin ($T_{1/2}$ = 6 hours) so that at 4 hours the values were 61% of control. At this time, the vitamin K_1 oxide/vitamin K ratio was 0.14 ± 0.03 which is a ratio which has been associated with the lack of an inhibitory effect by Bell and Matschiner (12).

The goal of the next experiment was to elevate vitamin K_1

TABLE I

EFFECTS OF WARFARIN ON HEPATIC K_1O/K_1 RATIOS AND PLASMA PROTHROMBIN LEVELS IN SPRAGUE-DAWLEY RATS GIVEN A PHYSIOLOGICAL DOSE OF ^{3}H-VITAMIN K_1*

Group	Time	Warfarin**	$K_{oxide/K}$	Prothrombin (% Control)	% DPM ^{3}H Extracted
1	2 Hr	-	0.04 ± 0.01	100 ± 2	32.3 ± 0.5
2	2 Hr	+	0.14 ± 0.03	79 ± 2	34.9 ± 1.5
3	4 Hr	-	0.04 ± 0.01	101 ± 3	29.7 ± 2.5
4	4 Hr	+	0.13 ± 0.03	61 ± 1	32.2 ± 1.7

* ^{3}H-K_1 infused at the rate of 50 ng/hr (1.5 μCi/hr). Each group contained 3-5 rats.

**Warfarin given at 1 mg/kg body weight at -30 minutes.

oxide/vitamin K levels in the liver to high values in the absence of warfarin to determine whether or not vitamin K_1 oxide administered alone could inhibit prothrombin synthesis. In order to achieve such high levels, we had to administer vitamin K oxide in high doses intermittently by vein over a 4 hour period. 400 μg of ^{3}H-vitamin K oxide (0.93 μCi/mg) were administered intravenously every 15 minutes for 3 hours and 45 minutes. At 4 hours, the livers were excised and the hepatic vitamin K_1 oxide/vitamin K ratio was determined. The results are shown in Table II. At the end of 4 hours, the vitamin K oxide/vitamin K ratio was 7.9 ± 1.5 in the normal rats and 10.9 ± 0.4 in warfarin resistant animals. The time course of hepatic vitamin K_1 oxide/vitamin K ratios is shown in Figure 1.

As can be seen from Table II and Figure 1, prothrombin levels did not change during the period of the experiment even though vitamin K oxide/vitamin K ratios were in excess of two

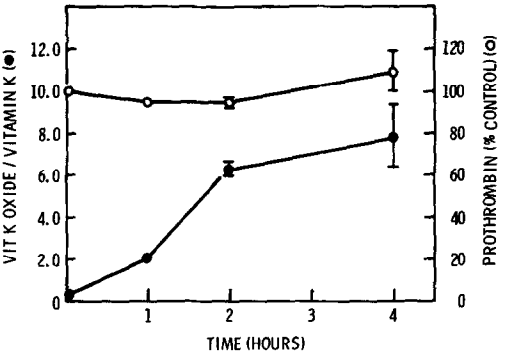

Figure 1. Effect of high concentrations of vitamin K oxide on prothrombin levels in the rat.

TABLE II

EFFECT OF INTERMITTENT INTRAVENOUS INFUSION OF ^{3}H-VITAMIN K OXIDE ON HEPATIC K_1O/K_1 RATIOS AND PROTHROMBIN LEVELS

Group	Vit. K_1 Oxide*	K_1O/K_1	Prothrombin (% Control)	% DPM Extracted
Sprague-Dawley	+	7.9 ± 1.5	110 ± 9	31.8 ± 3.4
Sprague-Dawley	-	-	109 ± 9	-
Warfarin Resistant	+	10.9 ± 1.4	104 ± 3	26.2 ± 1.3
Warfarin Resistant	-	-	103 ± 2	-

*400 µg of ^{3}H-vitamin K oxide was injected into the femoral vein every 15 minutes for a 4 hour period (1.5 µCi/hr). Each group contained 3-5 rats.

for a period of 3 hours which is sufficiently long to show an effect upon prothrombin levels. Although we have confirmed the original observation of Matschiner et al. (1) that warfarin inhibits the conversion of vitamin K oxide to vitamin K by inhibiting the reductase, we find no evidence for the action of the accumulated vitamin K oxide as an inhibitor of vitamin K.

In animals receiving minimal physiologic amounts of vitamin K in the presence of warfarin, there was an inhibition in prothrombin synthesis by the drug at vitamin K_1 oxide/vitamin K ratios in the normal range. On the other hand, the intermittent infusion of ^{3}H-vitamin K_1 oxide causes very high vitamin K_1 oxide/vitamin K ratios in the liver without any effect on prothrombin synthesis.

Suttie has shown that chlorophylloquinone, a competitive inhibitor of vitamin K_1, inhibits prothrombin synthesis in warfarin resistant rats (13) and Ren et al. (14) have shown that chlorophylloquinone inhibits prothrombin synthesis without appreciably affecting the hepatic vitamin K_1 oxide/vitamin K ratio. These data can all be reconciled by a simple hypothesis that provides for a receptor protein which has sites for both vitamin K and warfarin (5). We postulate further that the two ligands have antagonistic and reciprocal effects on the conformation of this protein which serves as a switch for prothrombin synthesis. Besides reacting with the receptor protein and modifying it to the off position, it is possible that warfarin's secondary effect on the reductase may restrict the resynthesis of vitamin K from its oxide and in that manner intensify the anticoagulant effect.

ACKNOWLEDGMENTS

This research was supported in part by grants AM 09992 and GM 446 from the National Institutes of Health, U.S.P.H.S., Bethesda, Maryland.

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