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Mechanism of Warfarin Resistance. Warfarin and the Metabolism of Vitamin K_1†

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ABSTRACT: If warfarin exerts its anticoagulant effect by causing an accumulation of phylloquinone oxide, an inhibitor of vitamin K, resistance to warfarin could be due to a mutation which renders the conversion of the oxide to vitamin K less sensitive to warfarin. Metabolic studies with ^{3}H-labeled vitamin K_1 and [^{3}H]phylloquinone oxide have provided evidence that resistance is due to the inability of the anticoagulant to inhibit the oxide–K_1 conversion. Homozygous resistant rats had elevated vitamin K requirements and were resistant to warfarin at 0.3 mg/100 g body weight but prothrombin synthesis was blocked by 5 mg/100 g body weight. Phylloquinone oxide stimulated prothrombin synthesis in resistant rats given warfarin but was ineffective in Sprague-Dawley rats treated with anticoagulant. In Sprague-Dawley rats warfarin (0.1 mg/100 g body weight) increases the oxide:K_1 ratio and decreases the concentration of vitamin K_1 in the liver but in resistant animals given ^{3}H-labeled vitamin K_1 the same dose of warfarin did not increase the oxide:K_1 ratio and had little effect on the amount of ^{3}H-labeled vitamin K_1 in the liver. In Sprague-Dawley rats given [^{3}H]phylloquinone oxide, warfarin almost completely blocks the conversion of [^{3}H]-phylloquinone oxide to ^{3}H-labeled vitamin K_1 and increases the oxide:K_1 ratio and the amount of [^{3}H]phylloquinone oxide in the liver. However, in resistant rats injected with [^{3}H]phylloquinone oxide, warfarin had little effect on the oxide:K_1 ratio or on the amount of unmetabolized [^{3}H]-phylloquinone oxide in the liver. If a dose of warfarin (5 mg/100 g body weight) sufficient to block prothrombin synthesis was administered to resistant rats, the conversion of [^{3}H]-phylloquinone oxide to tritiated vitamin K_1 was clearly inhibited and the amount of unmetabolized [^{3}H]phylloquinone oxide in the liver increased. The results are consistent with the idea that resistance is due to a mutation which alters the enzyme system which converts oxide to K_1 so that it is no longer inhibited by warfarin. The metabolic studies also suggest that the altered enzyme was less effective in catalyzing the oxide–K_1 conversion which could account for the high vitamin K requirements. However, the oxide was as effective as K_1 in stimulating prothrombin synthesis in hypoprothrombinemic resistant rats.

The mechanism of warfarin resistance is of great interest because of the spread of warfarin-resistant rats in Europe and the United States (Greaves, 1970; Jackson and Kaukeinen, 1972) and because of the insight it may give into the mechanism of action of the anticoagulant. Coumarin anticoagulants are not only important rodenticides but are widely used in the treatment of thromboembolic disease. Vitamin K_1, whose stimulus of clotting protein synthesis is blocked by warfarin, shuttles between the active vitamin and an inactive metabolite, phylloquinone oxide, in the liver (Bell and Matschiner, 1970, 1972; Matschiner *et al.*, 1970; Bell *et al.*, 1972). Warfarin traps the vitamin as the inactive oxide by inhibiting its conversion back to vitamin K_1. We have proposed that warfarin exerts its effect by increasing the concentration of oxide which acts as an inhibitor of the vitamin (Bell and Matschiner, 1972) and predicted that resistance to warfarin could be due to a mutation which renders the conversion of the oxide to vitamin K_1 insensitive to warfarin. Zimmerman and Matschiner (1972) have reported that the conversion of phylloquinone oxide to vitamin K_1 by liver preparations is in-

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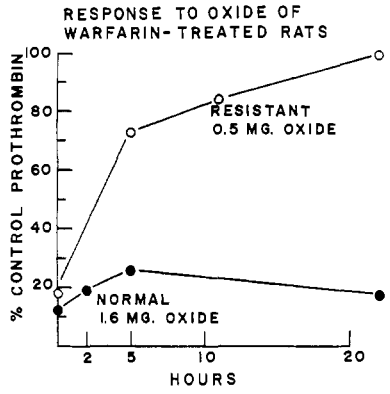

FIGURE 1: Response of warfarin-treated rats to phylloquinone oxide. (○) Resistant rats were injected intraperitoneally with sodium warfarin (0.15 mg/100 g body weight) 2 hr before intramuscular injection with 0.5 mg of phylloquinone oxide in Tween solution at zero time. (●) Sprague-Dawley rats were injected with sodium warfarin (0.15 mg/100 g body weight) 24 hr before intramuscular injection of 1.6 mg of phylloquinone oxide. Each point represents the average of three rats.

hibited by warfarin *in vitro* and the inhibition is reduced in preparations from resistant rats (Zimmerman and Matschiner, 1972). The present paper provides evidence from *in vivo* studies that warfarin resistance is due to the inability of the anticoagulant to prevent the oxide–K_1 conversion, thus preventing the accumulation of sufficient oxide to inhibit clotting protein synthesis.

Materials and Methods

Warfarin-resistant rats were obtained from Dr. Judith Pool (Department of Medicine, Stanford University) who crossbred wild resistant rats captured in England with normal Sprague-Dawley rats. The resistant descendants were inbred to produce rats homozygous for the resistant trait (Pool *et al.*, 1968). We bred resistant rats with one another and used them at 10–15 weeks of age in the following experiments.

Because of the greater requirement of vitamin K of resistant animals (Hermodson *et al.*, 1969), pregnant rats were given menadione sodium bisulfite (1 mg/l.) in the drinking water (Ernster *et al.*, 1972) in order to increase the number of survivors in the litters obtained. After weaning the rats did not receive any vitamin K supplement.

The control rats in these experiments were Sprague-Dawley males (10–14 weeks old) of the CD strain from Charles River Laboratories. Both resistant and control animals were fed Purina rat chow. Prothrombin was assayed by the method of Hjort *et al.* (1955). The control plasma was pooled plasma from 20 11–12-week old male Sprague-Dawley rats.

Phylloquinone oxide was prepared from vitamin K_1 (Nutritional Biochemical Corporation) according to Tishler *et al.* (1940).

6,7-Tritiated vitamin K_1 and tritiated phylloquinone oxide were generous gifts from Dr. J. T. Matschiner and were purified as previously described (Matschiner *et al.*, 1970). They were dissolved in Tween 80 and diluted with 0.9% NaCl to make solutions containing 5% Tween or less; 0.1 or 0.2 ml was injected intracardially.

Portions of liver were analyzed as previously described (Bell *et al.*, 1972). Hexane extracts were chromatographed on silicic acid which separates vitamin K_1 and phylloquinone oxide from more polar metabolites. The vitamin and oxide were separated with carrier by thin layer chromatography on commercially prepared plates of silica gel (Brinkmann Instruments, Inc., Westbury, N. Y.) coated with liquid paraffin. The developing solvent was a mixture of acetone and water (92:8). Spots were visualized by ultraviolet absorption and iodine vapor and counted directly in a liquid scintillation spectrometer.

TABLE I: Warfarin Resistance.[a]

	No. of Rats	Warfarin (mg/100 g Body Wt)	% Control Prothrombin: Zero Time	16 hr after K_1	8 hr after Warfarin
Male resistant	16	0.3	17	108	120
Male resistant	3	5.0	17	108	49
Male Sprague-Dawley	3	0.3	100	105	50
Female resistant	3	0.3	43	107	130
Female resistant	3	5.0	55		25

[a] In all experiments except the last, rats were injected intramuscularly at zero time with 30 μg/100 g body weight of vitamin K_1 and 16 hr later they were injected intraperitoneally with sodium warfarin. In the last experiment the female resistant rats were injected only with warfarin (5 mg/100 g body weight) and blood samples were taken 8 hr later.

Results

Warfarin Resistance. Male resistant rats fed Purina rat chow had prothrombin levels of 17% of control indicating a higher vitamin K requirement than Sprague-Dawley animals, as previously observed by Hermodson *et al.* (1969). Female resistant rats also had lowered plasma prothrombin levels (52% of control) but significantly above those of males. It is well known that female rats have lower vitamin K requirements than males (Metta and Johnson, 1960).

To test the offspring of resistant rats for warfarin resistance, they were injected with vitamin K_1 (30 μg/100 g body weight) to increase plasma prothrombin to normal levels and then given 0.3 mg/100 g body weight of warfarin (Table I). In both male and female resistant animals, the prothrombin concentration was not reduced 8 hr after warfarin, but in Sprague-Dawley rats prothrombin decreased with a half-life of about 8 hr. The half-life of prothrombin in male rats from the St. Louis University colony treated with warfarin (1 mg/100 g body weight) was found previously to be 7 hr (Bell and Matschiner, 1969). If the dose of warfarin was increased to 5 mg/100 g body weight, prothrombin synthesis was blocked in both male and female resistant rats.

Response of Warfarin-Treated Rats to Phylloquinone Oxide. Phylloquinone oxide is as effective as vitamin K_1 in stimulating prothrombin synthesis in Sprague-Dawley rats fed vitamin K deficient diets, but is ineffective against warfarin (Bell and Matschiner, 1970). However, in resistant rats treated with warfarin (0.15 mg/100 g body weight), 0.5 mg of oxide increased prothrombin levels at a rate similar to vitamin K_1 (Bell *et al.*, 1972) and restored normal prothrombin in 22

TABLE II: Metabolism of Tritiated Vitamin K_1 and Phylloquinone Oxide in Sprague-Dawley and Resistant Rats.[a]

	Warfarin (mg/100 g Body Wt)	% of Injected ^3H at 2 hr		
		In Liver	In Tritiated Vitamin K_1	Oxide:K_1
Injected with tritiated vitamin K_1				
Sprague-Dawley	—	38	9.9 ± 2.2	0.11 ± 0.02
	0.1	35	5.5 ± 1.2	0.89 ± 0.12
Resistant	—	29	10.9 ± 1.8	0.36 ± 0.09
	0.1	18	8.7 ± 0.3	0.24 ± 0.04
Injected with [^3H]phylloquinone oxide			In [^3H]Phylloquinone Oxide	
Sprague-Dawley	—	34	5.8 ± 1.3	0.57 ± 0.16
	0.1	51	14.1 ± 1.3	19.0 ± 2.9
Resistant	—	27	8.7 ± 1.3	1.37 ± 0.14
	0.1	25	9.0 ± 2.4	1.70 ± 0.61
	5.0	38	16.0 ± 1.0	8.10 ± 1.76

[a]Male rats were injected intracardially with 0.1 mg of tritiated vitamin K_1 or [^3H]phylloquinone oxide and livers were removed 2 hr later, frozen, and later analyzed as described previously (Bell *et al.*, 1972). Sodium warfarin where indicated was injected intraperitoneally 24 hr before the labeled vitamin or oxide. The 5.0 mg/100 g body weight dose of warfarin was injected 0.5 hr before the radioactive oxide. The results are the averages for three–five rats ± the standard errors of the means. Radioactivity was assayed in a liquid scintillation counter. The results for Sprague-Dawley rats are taken from Bell *et al.* (1972).

hr (Figure 1). In contrast, a dose of oxide three times larger had no effect on plasma prothrombin in Sprague-Dawley rats treated with warfarin (0.15 mg/100 g body weight) 24 hr previously. This suggests that warfarin at this level does not prevent the reduction of phylloquinone oxide to vitamin K_1 in resistant animals.

Metabolism of ^3H-Labeled Vitamin K_1 and [^3H]Phylloquinone Oxide in Resistant Rats. To test this idea further, the metabolism of tritiated vitamin K_1 and [^3H]phylloquinone oxide was studied in the presence and absence of warfarin in resistant rats and compared to previous results obtained when identical experiments were done in Sprague-Dawley rats (Bell *et al.*, 1972) (Table II). In Sprague-Dawley rats given ^3H-labeled vitamin K_1, warfarin (0.1 mg/100 g body weight) increased the oxide:K_1 ratio and reduced the amount of ^3H-labeled vitamin K_1 in the liver at 2 hr. However, in resistant animals the same dose of warfarin did not increase the oxide:K_1 ratio and had little effect on the amount of ^3H-labeled vitamin K_1 in the liver. In Sprague-Dawley rats [^3H]phylloquinone oxide was extensively converted to vitamin K_1 but warfarin almost completely blocked the conversion resulting in a concentration of [^3H]phylloquinone oxide three times higher than in the untreated rats. However, in resistant rats warfarin had little effect on the oxide:K_1 ratio or on the amount of unmetabolized [^3H]phylloquinone oxide remaining after 2 hr. If a dose of warfarin (5 mg/100 g body weight) sufficient to block prothrombin synthesis was administered to resistant rats 0.5 hr before [^3H]phylloquinone oxide, the conversion of oxide to K_1 was clearly inhibited. The oxide:K_1 ratio was almost five times greater than in resistant rats given an ineffective dose of warfarin (0.1 mg/100 g body weight) 24 hr before [^3H]phylloquinone oxide. The amount of [^3H]phylloquinone oxide in the liver was similar to that found when Sprague-Dawley rats were treated with 0.1 mg/100 g body weight of warfarin which is sufficient to block prothrombin synthesis in these animals.

Response of Hypoprothrombinemic Resistant Rats to Vitamin K_1 and Phylloquinone Oxide. The above results suggest that the enzyme catalyzing the oxide–K_1 conversion is altered in resistant animals so that it is no longer sensitive to warfarin. If the altered enzyme is less effective in the reduction of the oxide this might explain the increased vitamin K requirement in resistant rats. The concentration of vitamin would be reduced if the K_1–oxide conversion proceeded at the normal rate, but the oxide–K_1 conversion was slowed down sufficiently. Matschiner *et al.* (1973) have found that the activities of liver fractions which catalyze the oxide–K_1 conversion are reduced in resistant rats. This explanation is supported by data in Table II where after tritiated K_1 or tritiated oxide administration, the oxide:K_1 ratio was over twice as high in resistant animals with or without warfarin (0.1 mg/100 g body weight) as compared to Sprague-Dawley rats without the anticoagulant. On the other hand, the hepatic concentration of tritiated K_1 in the resistant rats was not significantly different from that occurring in the livers of Sprague-Dawley animals. If the oxide–K_1 conversion is impaired in resistant rats, phylloquinone oxide should be less effective than vitamin K_1 in stimulating prothrombin synthesis in resistant rats which have low prothrombin levels when fed Purina lab chow. However, over a range dose from 1 to 100 μg, the oxide was as effective as vitamin K_1 in increasing plasma prothrombin levels (Table III). The response of vitamin K deficient Sprague-Dawley rats to 1 μg of vitamin K_1 was similar to that observed in the resistant rats injected with 1 μg of K_1 or oxide. However, the resistant animals received vitamin in the Purina chow diet while the Sprague-Dawley rats were fed a synthetic diet devoid of vitamin K. This is consistent with a higher vitamin K requirement in resistant rats.

Discussion

The results clearly show that warfarin, at doses which cause the accumulation of phylloquinone oxide and block prothrombin synthesis in Sprague-Dawley rats, does not cause the accumulation of the oxide in resistant rats as demonstrated by metabolic experiments with ^3H-labeled vitamin K_1 and

TABLE III: Response of Resistant Rats to Vitamin K_1 and Phylloquinone Oxide.[a]

Injected	No. of Rats	% Control Prothrombin 0	2 hr	11 hr	16 hr
Resistant					
100 μg of K_1	16	17			108
100 μg of oxide	3	17			94
10 μg of K_1	3	17	49	94	
10 μg of oxide	3	17	40	88	
1 μg of K_1	3	17		30	
1 μg of oxide	4	17		47	
Sprague-Dawley					
1 μg of K_1	4	24		53	

[a] Male resistant rats fed Purina chow and male Sprague-Dawley rats fed a vitamin K deficient synthetic diet (Matschiner and Taggart, 1968) were injected intramuscularly with vitamin K_1 or phylloquinone oxide in aqueous Tween solutions. The average zero-time prothrombin concentration of resistant rats was 17 $\pm$ 2.5% (SE) of the control. The results are the average for the number of rats shown in the first column.

has little effect on the oxide-K_1 conversion as shown by experiments with [^{3}H]phylloquinone oxide (Table III). This indicates that high oxide:K_1 ratios, which occur when the syntheses of clotting proteins are inhibited by warfarin in laboratory rats (Matschiner *et al.*, 1970; Bell *et al.*, 1972), do not occur in resistant animals. A high dose of warfarin (5 mg/100 g body weight) which blocks prothrombin synthesis even in resistant rats (Table I) will also inhibit the oxide-K_1 interconversion (Table II). The results are consistent with the idea that resistance is due to a mutation which alters the enzyme system which converts phylloquinone oxide to vitamin K_1 so that it is no longer inhibited by warfarin.

Hermodson *et al.* (1969) and O'Reilly (1970, 1971) have proposed that warfarin resistance results from a mutation that causes the synthesis of a protein with a lowered binding affinity for both vitamin K and warfarin. This is supported by the high vitamin K requirement in both warfarin resistant rats (Hermodson *et al.*, 1969) and humans (O'Reilly, 1971) and the decreased ability of microsomes from resistant rats to bind warfarin *in vitro* (Lorusso and Suttie, 1972). The reduced binding of warfarin in microsomes from resistant rats could be explained by our hypotheses since the enzyme system which converts phylloquinone oxide to vitamin K_1 is found in the microsomal fraction (Zimmerman and Matschiner, 1972). The high vitamin K requirement could also be explained if the altered enzyme system was less effective in converting the oxide back to vitamin K_1 in the absence of warfarin. However, phylloquinone oxide was as effective as vitamin K_1 in stimulating prothrombin synthesis in hypoprothrombinemic resistant rats (Table III).

Sprague-Dawley rats appear to be adequate controls for these studies since the resistant rats were mated with this strain for six generations which would result in a frequency of incrosses of 95% (Pool *et al.*, 1968). A number of studies have revealed little difference in the metabolism of warfarin, vitamin K_1, or menadiol phosphate in resistant and laboratory rats (Pool *et al.*, 1968; Hermodson *et al.*, 1969; Thierry *et al.*, 1970; Davies and Davies, 1970). Ernster *et al.* (1972) found that the activities of a variety of liver enzymes were similar in resistant and Sprague-Dawley rats but the activity of DT-diaphorase was ten times lower in resistant animals. The enzymes from both strains were inhibited by coumarin anticoagulants in a similar fashion. It was suggested that DT-diaphorase serves to convert oxidation products of vitamin K back to the vitamin and a decrease in its activity would lead to vitamin K deficiency in resistant and coumarin-treated normal rats. Although warfarin lowered the hepatic level of ^{3}H-labeled vitamin K_1 in Sprague-Dawley rats treated with warfarin (Table II) this is not likely to be the primary way in which coumarins exert their effect because the concentration would have to be decreased to a much greater extent. As much as 25 μg of vitamin K_1 is ineffective in overcoming warfarin inhibition of prothrombin synthesis (Bell *et al.*, 1972), whereas it requires only about 0.5 μg of vitamin K_1 to maximally stimulate prothrombin synthesis in vitamin K deficient rats (Matschiner and Taggart, 1968).

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