Evidence That Warfarin Anticoagulant Action Involves Two Distinct Reductase Activities*

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Michael J. Fasco‡§, Ellen F. Hildebrandt¶, and John W. Suttie**

From the ‡Center for Laboratories and Research, New York State Department of Health, Albany, New York 12201 and the ¶Department of Biochemistry, College of Agricultural and Life Sciences, University of Wisconsin-Madison, Madison, Wisconsin 53706.

The dithiothreitol-dependent vitamin K and vitamin K 2,3-epoxide hepatic microsomal reductase activities of warfarin-susceptible and warfarin-resistant rats were compared to gain insight into the role(s) of these activities in vitamin K metabolism and function. In microsomes from resistant rats, 3- to 4-fold more warfarin was required to produce 50% inhibition (I₅₀) of vitamin K reduction to vitamin K hydroquinone than in microsomes from susceptible rats. For the reduction of vitamin K 2,3-epoxide to vitamin K a 6-fold higher warfarin concentration was required. In microsomes from resistant rats, the I₅₀ warfarin concentration required to inhibit γ-carboxylation of microsomal precursor protein was also 4-fold higher with vitamin K as substrate and was 6-fold higher with the epoxide as substrate in microsomes from susceptible rats. Collectively, these data suggest that the vitamin K reductase contributes to the metabolism of vitamin K in intact rats and that warfarin inhibition of both the vitamin K and vitamin K 2,3-epoxide reductases is involved in its anticoagulant effect.

Vitamin K is required for the post-translational formation of γ-carboxyglutamyl residues from Glu residues in a limited number of proteins including clotting Factors II, VII, IX, and X (1). The O₂-dependent microsomal carboxylase responsible for this carboxylation event requires the reduced form of vitamin K, vitamin KH₂,¹ as a cofactor (2), and current evidence suggests that vitamin K 2,3-epoxide may be a product of this reaction (3). Vitamin KH₂ formation from vitamin K may be catalyzed by DT-diaphorase (EC 1.6.99.2) (4, 5) and/ or an uncharacterized microsomal reductase which can utilize dithiothreitol as cofactor in vitro (6).

Warfarin and many other 4-hydroxycoumarin drugs are potent inhibitors of vitamin K-dependent protein formation and have consequently found extensive use in clinical medicine and as rodenticides. Warfarin causes an accumulation of vitamin K 2,3-epoxide in liver and strongly inhibits a microsomal vitamin K 2,3-epoxide reductase (7).

The discovery of rats resistant to the anticoagulant effects of warfarin (8) has made available a valuable tool to study the action of this drug. Metabolism of warfarin or vitamin K is apparently not altered in these animals (9-11), although differences in warfarin binding to an unidentified protein in the microsomes have been demonstrated (12, 13). The vitamin K 2,3-epoxide reductase of warfarin-resistant rats is much less sensitive to inhibition by warfarin than is the reductase of susceptible rats (14), but the DT-diaphorase, vitamin K-dependent carboxylase, and vitamin K epoxidase activities are not (15, 16). These data support the conclusion that the genetic alteration which confers resistance is highly selective and affects only vitamin K 2,3-epoxide reductase (6, 15, 16).

The microsomal dithiothreitol-dependent, warfarin-sensitive vitamin K reductase described by Whitlon et al. (6) has not been investigated as thoroughly as other enzymes of the vitamin K metabolic cycle, partially because of difficulties associated with quantification of the vitamin KH₂ product of the reaction. Recently, an assay has been developed which quantifies microsomal vitamin KH₂ formation with either vitamin K or its 2,3-epoxide as substrate (17). We report here two separate lines of evidence which suggest that the anticoagulant action of warfarin is due to inhibition of the reduction of vitamin K as well as of vitamin K 2,3-epoxide.

EXPERIMENTAL PROCEDURES

Warfarin-susceptible Holtzman male rats (Holtzman Company, Madison, WI) and warfarin-resistant male rats (from a breeding colony maintained at the University of Wisconsin-Madison) were all fed Purina Lab Chow. Resistant rats were provided 1 mg of menadione sodium bisulfite/liter of drinking water. The rats were fasted overnight prior to decapitation. All further operations were performed at 5 °C.

The livers excised from five susceptible or five resistant rats were pooled, rinsed with 0.02 M Tris-HCl, 0.25 M sucrose buffer (pH 7.4), minced, and homogenized with a Potter-Elvehjem homogenizer in two volumes (by weight) of the same buffer. The cellular debris, nuclei, and mitochondria were removed by centrifugation at 10,000 × g for 10 min and the microsomes were sedimented from the supernatant solution by centrifugation at 105,000 × g for 60 min. The microsomes were resuspended by homogenization in the Tris-sucrose buffer, and aliquots were frozen at −80 °C until use. Microsomal protein concentrations were determined by the method of Bradford (18), with the use of Bio-Rad reagents and bovine serum albumin as standard.

Vitamin K₂ (Sigma) was used without further purification. Vitamin K 2,3-epoxide was prepared by hydrogen peroxide oxidation of vitamin K and was purified by HPLC (19). Vitamin KH₂ was prepared by dithiothreitol reduction of vitamin K at pH 8.5 (17), and warfarin (Calbiochem) sodium salt was prepared by the method of West et al. (20). Aqueous Emulgen 911 (Kao Atlas, Tokyo, Japan) solutions (9.1 v/v) of vitamin K or vitamin K 2,3-epoxide (30 mg/ml) were prepared as described previously (17) and were diluted with water for use in metabolism studies.

Reaction mixtures contained, in order of addition: 1.35 ml of 200 mM Tris-HCl, 0.15 M KCl buffer (pH 7.4) with or without warfarin
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sodium salt; 0.6 ml of microsomes (10 mg of protein/ml); and 0.01 ml of vitamin K or vitamin K 2,3-epoxide (2 mg/ml). Each mixture was incubated at 25 °C with gentle agitation for 1 min, after which the reaction was initiated by addition of 0.04 ml of diethiothreitol solution (100 mM). After incubation at 25 °C for an additional 5 min, vitamin substrates and metabolites were extracted with 4 ml of isopropanol:hexane (1:1) by vortex mixing for 20 s. After brief, low speed centrifugation, a 1.5-ml aliquot of the solvent phase was evaporated to dryness at 30 °C under oxygen-free nitrogen, and the residue was redissolved in 0.2 ml of isopropanol.

The vitamin components were separated by HPLC as described (17), except that the column was a Waters RCM C18 (5 mm, inner diameter), solvent A was water-acetonitrile:isopropanol (6:4:1) and solvent B was acetonitrile:isopropanol (4:1). Compounds were detected at 254 nm and were quantitated by integrated area comparison with external standards. Variations of this method are described in the figure legends.

Warfarin Inhibition

Warfarin was a potent inhibitor of the conversion of vitamin K to vitamin KH₂ in microsomal preparations from susceptible rats. The I₅₀ for this reaction was 1 µM warfarin, compared to 3 to 4 µM for resistant rat microsomes (Fig. 1A). Warfarin also inhibited the conversion of vitamin K 2,3-epoxide to vitamin K and vitamin KH₂ more effectively in susceptible than in resistant rat microsomes (Fig. 1B); the I₅₀ warfarin concentrations were approximately 2 and 12 µM, respectively. These findings clearly demonstrate that (a) warfarin not only acts at the epoxide reduction step but can also inhibit reduction of vitamin K and (b) both reactions are less sensitive to warfarin in the resistant rat.

Numerous in vitro studies have indicated that vitamin KH₂ formation is a physiologically relevant step in vitamin K-dependent carboxylation (1). With respect to inhibition by warfarin, the vitamin K-supported carboxylation activity in microsomes from resistant rats required a 4-fold higher I₅₀ warfarin concentration than did microsomes from susceptible rats (Fig. 2A); for the vitamin K epoxide-dependent reaction, the difference in sensitivity was about 6-fold (Fig. 2B). These differences between microsomes of the two strains were thus in good agreement with differences obtained by the direct vitamin KH₂ quantitation method. The actual I₅₀ warfarin concentrations for epoxide metabolism or epoxide-driven carboxylation were also in good agreement, being for each assay method approximately 2 µM in susceptible and 12 µM in resistant rat microsomes.

The I₅₀ warfarin concentrations for vitamin K reduction, however, were approximately 12-fold lower than those for vitamin K-dependent carboxylation (1 versus 12 µM in susceptible and 3–4 versus 47 µM in resistant rat microsomes). A possible explanation for this difference is that in the presence of excess vitamin K substrate, the KH₂ formation rate is greatly in excess of that required for optimum activity of the carboxylase. The higher warfarin concentrations required for 50% inhibition in this assay reflect that difference. When vitamin K epoxide is used as substrate, two reduction steps are involved in generating the active hydroquinone form of the vitamin and its formation rate may be limiting for the carboxylase. Indeed, relative to vitamin K 2,3-epoxide, vitamin K promoted the incorporation of much higher concentrations of ¹⁴CO₂ into micromolar precursor proteins of either rat strain (Fig. 2 legend). Whatever the cause of the differences in the I₅₀ warfarin concentrations in the two assay methods, the data of both experiments support the concept that both vitamin K and vitamin K 2,3-epoxide reduction are warfarin-sensitive and altered in the resistant rat.

With microsomes from both rat strains, metabolism of vitamin K epoxide to vitamin K and vitamin KH₂ as a function of time (Fig. 3) demonstrated that the only product formed during the initial 2 min was vitamin K. Thereafter, vitamin K formation attained a steady state rate, and the concentration of vitamin KH₂, the second metabolite of the reaction, increased. These data are consistent with those obtained for microsomes of Wistar rats (22) and suggest that the reaction sequence is vitamin K epoxide to vitamin K to vitamin KH₂, where vitamin K must attain a minimum concentration before it can serve as a substrate for further reduction. After this concentration is achieved, the vitamin KH₂ formation rate becomes equal to the vitamin K formation rate, preventing further accumulation of vitamin K. Vitamin K epoxide metabolism to vitamin KH₂ is therefore an ordered process in which vitamin K must interact at a site either within the same enzyme or on another enzyme prior to its reduction to hydroquinone.

Microsomes of susceptible and resistant rats had similar profiles of vitamin K 2,3-epoxide metabolism to vitamins K and KH₂ over the time span investigated, although higher
metabolite concentrations were produced by microsomes of susceptible rats at the 20-μM concentration of vitamin K epoxide used. Warfarin slows the rate of vitamin KH₂ formation both by decreasing the concentration of available vitamin K and by inhibiting the conversion of vitamin K to vitamin KH₂. The combined effect produces a pronounced decrease in vitamin KH₂ formation (Fig. 3A).

In microsomes from resistant rats, 3 μM warfarin did not inhibit vitamin K formation from the epoxide as completely as did 1 μM warfarin in microsomes from susceptible rats. Thus, the microsomes of resistant rats have higher concentrations of vitamin KH₂. Since the conversion of vitamin K to KH₂ in these microsomes is also less sensitive to inhibition by warfarin, quantities of vitamin KH₂ are further increased, relative to those in microsomes of susceptible rats. Thus, after 15 min of reaction, 1 μM warfarin in microsomes of resistant rats produced an 84% reduction of vitamin KH₂ formation, while 3 μM warfarin in microsomes of resistant rats reduced vitamin KH₂ formation by only 26%. If the assumption is made that both the vitamin K and vitamin K 2,3-epoxide reductase activities are normally required to maintain tissue vitamin KH₂ concentrations, warfarin insensitivity at both sites will confer greater resistance than would be predicted from the extent of inhibition of either reaction. Indeed, relative to susceptible rats, resistant rats are 50 to 200 times less sensitive to warfarin.

Previous knowledge regarding the importance of vitamin K 2,3-epoxide reductase as the physiologically important site of action of warfarin has been derived from comparisons of vitamin K metabolism in susceptible and resistant rats. The evidence presented here clearly indicates that vitamin KH₂ arises from vitamin K 2,3-epoxide through a vitamin K intermediate and that this second reduction step is both warfarin-sensitive and altered in the resistant rat. The physiologic importance of the NADH-dependent reductases of microsomes cannot be determined from these data, but both dihydrothreitol-dependent steps are clearly involved in the action of warfarin as an inhibitor of vitamin K-dependent reactions.

The nature of the physiologic reductant replaced by dihydrothreitol in these reactions is not known. Neither do these data clearly indicate whether both reductive steps are catalyzed by the same site on a single enzyme, by separate sites on a single enzyme, or by two enzymes. The low probability of a genetic alteration affecting two enzymes might argue against the last possibility unless there is some protein-protein interaction between these enzymes. A clear understanding of the factors which mediate vitamin K and vitamin K 2,3-epoxide reduction will probably come only from efforts to purify these membrane-bound activities.

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