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BIOCHEMICAL BASIS OF HEREDITARY RESISTANCE TO WARFARIN IN THE RAT

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Abstract—The influence of warfarin on prothrombin formation and the regeneration of vitamin K from vitamin K-2,3-epoxide was examined in control and warfarin-resistant rats. The epoxide reductase from liver of resistant rats was inhibited by warfarin less than the reductase from control animals. The concentration of warfarin required to inhibit the reductase *in vitro* was similar in control and resistant rats to the concentration of warfarin *in vivo* during inhibition of prothrombin formation. These results are interpreted as evidence of the biochemical basis for hereditary resistance to warfarin in the rat.

RECENT EVIDENCE suggests that warfarin inhibits the synthesis of prothrombin by causing the accumulation of a natural metabolite inhibitory to vitamin K[1,2]. This metabolite, a naphthoquinone (vitamin K)-2,3-epoxide, is reduced to vitamin K by a system from rat liver which is not fully characterized but which is membrane-bound and stimulated by thiol compounds.[3] In the presence of warfarin, the reductase is inhibited, resulting in accumulation of the epoxide.[4]

The mechanism of hereditary resistance to warfarin in the rat has been studied in several laboratories. In terms of the classic theory of warfarin action, resistance has been proposed to result from lowered affinity of a hypothetical receptor protein for both vitamin K and warfarin.[5] Because of the opportunity these mutants afforded to test the new hypothesis for the mechanism of action of warfarin, we undertook a study of the reduction of vitamin K epoxide by liver from resistant animals. While these studies were in progress, a similar report appeared implicating DT-diaphorase[6] in the action of warfarin and hereditary resistance to the drug.

MATERIALS AND METHODS

Radioactive compounds. Phylloquinone-6,7-^{3}H was prepared from tritiated menadione.[7] Radioactive epoxide was synthesized from the labeled vitamin by oxidation with hydrogen peroxide in aqueous ethanol containing sodium carbonate.[8] The product was purified by chromatographic methods described previously.[9]

^{14}C-Warfarin, 3α-acetonyl(benzyl)-α ^{14}C)-4-hydroxycoumarin, was purchased from Amersham-Searle, Arlington Heights, Ill. Radiochemical purity was confirmed by descending paper chromatography in *n*-butanol–ammonia–water (9:1:2), and by thin-layer chromatography in ethylene dichloride–acetone (9:1) and toluene–ethyl formate–formic acid (5:4:1). The ^{14}C-warfarin was identified spectrophotometrically in 0·1 M sodium biocarbonate by comparison of its spectrum with that of an authentic sample of sodium warfarin ($E_{1\,cm}^{1\%}$ 400 at 308 nm) obtained from Endo Laboratories, Garden City, N.Y. The sp. act. of the ^{14}C-warfarin was 6·48 mCi/m-mole.

Ultraviolet measurements were carried out in a Cary 15 spectrophotometer. Radioactivity was measured in a Packard 3375 liquid scintillation spectrometer with external standardization.

Animals. Homozygous warfarin-resistant adult male rats were obtained by sibling mating from rats supplied by Dr. Judith Pool at Stanford University. Control adult male rats were obtained from a local supplier.*

Studies in vitro. Homogenates of liver were prepared in 3 vol. of 0·05 M Tris, 0·16 M KCl, pH 7·4. Each incubation contained cytosol and 10,000 *g* pellet equivalent to 0·5 g liver in a total volume of 3 ml of the homogenizing medium. ^{3}H-phylloquinone epoxide was added in 50 μl of ethanol to start the reaction. Incubations were terminated by the addition of 2 vol. of propanol-2 hexane (1:1). The product of the reaction in the hexane extract was separated with carrier by thin-layer chromatography on Silica gel inpregnated with liquid paraffin.[9] The developing solvent was acetone:water (92:8).

Studies in vivo. Control and warfarin-resistant rats were injected with ^{14}C-warfarin, and the concentration of radioactive drug was measured in liver during inhibition of prothrombin synthesis and when the drug was present at ineffectual levels. Because of the high requirement for vitamin K in resistant rats,[5] these rats were fed a diet† supplemented with 10 μg of phylloquinone/g. One group of control rats was also fed the diet supplemented with vitamin K. Other control rats were fed Purina Laboratory Chow.

Rats were fasted 8–12 hr before sacrifice. Blood was taken by heart puncture, and plasma prothrombin was measured by the method of Hjort *et al.*[10] using Russell's viper venom as an extrinsic activator. The animals were killed by decapitation, the livers removed and aliquots taken immediately for radioassay. All tissues, homogenates and subcellular fractions were prepared for counting by combustion in a Packard model 305 sample oxidizer or by solubilization in Nuclear–Chicago solubilizer at 37° overnight.

For determination of warfarin, livers were homogenized in 3 vol. of 0·25 M sucrose, 0·025 M phosphate, pH 7·5, using a Polytron 20ST (Brinkmann Instruments, Inc.). Fractions were prepared using an RC2-B Sorvall centrifuge and an L3-50 Beckman ultracentrifuge. The microsomal pellet was fractionated on a discontinuous sucrose density gradient according to Dallner *et al.*[11] Warfarin was extracted from acidified portions of the whole homogenate and subcellular fractions with ether. The extract was chromatographed with carrier warfarin on thin-layer plates using ethylene dichloride–acetone (9:1) as the developing solvent. The location of warfarin was visualized in ultraviolet light after short exposure of the developed plate to ammonia. The amount of warfarin in each extract was calculated from the ^{14}C recovered as warfarin and the specific activity of the administered drug.

RESULTS

The conversion of vitamin K epoxide to vitamin K by liver from control and warfarin-resistant rats is shown in Table 1. The reaction was stimulated by the addition

* Sprague–Dawley derived Sasco rats, Omaha, Nebr.

† Purified diet deficient in vitamin K obtained from General Biochemicals, Inc., Chagrin Falls, Ohio.

TABLE 1. CONVERSION OF VITAMIN K EPOXIDE TO VITAMIN K AND INHIBITION BY WARFARIN IN CONTROL AND WARFARIN-RESISTANT RATS

Additions	Control rats (% conversion)*		Resistant rats (% conversion)*	
	15 min	30 min	15 min	30 min
None†	8	10	11	12
Warfarin‡	1	1	4	6
0·5 mM DTT	27	38	21	19
0·5 mM DTT + warfarin‡	16	15	20	17
2·0 mM DTT	29	40	19	19
2·0 mM DTT + warfarin‡	20	28	21	18

* The results are the average of duplicate incubations. Each incubation contained cytosol and 10,000 *g* pellet equivalent to 0·5 g liver in a total volume of 3 ml prepared as described in Materials and Methods, and 2·0 nmoles (1·5 × 10^6 dis./min) of ^{3}H-phylloquinone epoxide.

† The concentration of sulfhydryl in these incubations was 1·2 mM from added cytosol.[12] Each incubation contained 70 mg protein.[13]

‡ 3·5 μM.

of dithiothreitol (DTT) and inhibited by warfarin. The reductase from resistant rats was less sensitive to warfarin than the reductase from control rats under all the conditions shown. The reductase from both strains of rats was inhibited less by warfarin after the addition of DTT. The concentration of endogenous sulfhydryl in all incubations was 1·2 mM from added cytosol.

The results of a similar experiment at several concentrations of warfarin are shown in Fig. 1. At 2·0 mM DTT, inhibition of the reductase was incomplete at the highest concentration of warfarin tested. The difference in sensitivity of the reductase to warfarin between the strains of rat shown in Table 1 was confirmed by the results shown in Fig. 1.

Inhibition of vitamin K epoxide reductase at lower concentrations of warfarin in the absence of DTT is shown in Fig. 2. Maximum inhibition of reductase activity from

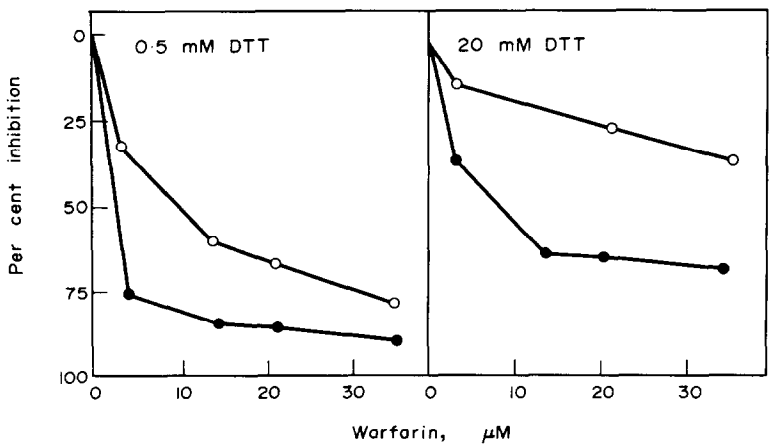

FIG. 1. Effect of dithiothreitol (DTT) on warfarin inhibition of phylloquinone epoxide reductase activity from livers of control (●) and warfarin-resistant (○) rats. The incubation mixtures contained cytosol and 10,000 *g* pellet equivalent to 0·5 g liver prepared as described in the text in a total volume of 3 ml. The substrate was 1 μg of ^{3}H-phylloquinone epoxide added in 50 μl of ethanol.

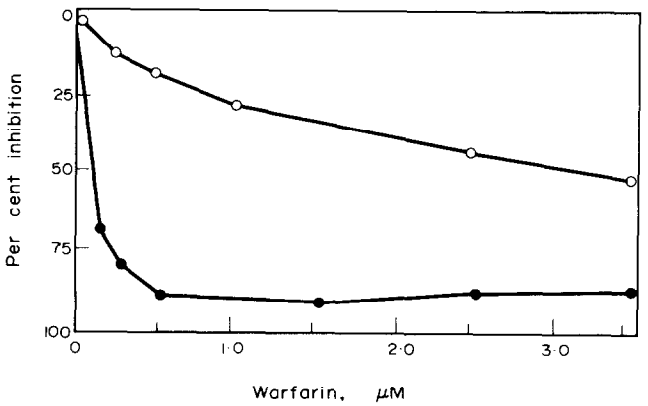

FIG. 2. Warfarin inhibition of phylloquinone epoxide reductase activity from livers of control (●) and warfarin-resistant (○) rats. The incubation mixtures contained cytosol and 10,000 *g* pellet equivalent to 0·5 g liver prepared as described in the text in a total volume of 3 ml. The substrate was 1 μg of ^{3}H-phylloquinone epoxide in 50 μl of ethanol.

control rats occurred with 0·5 μM warfarin. The reductase from liver of warfarin-resistant rats was less sensitive; only 50 per cent inhibition occurred in the presence of 3 μM warfarin. In an extension of these studies, about 90 per cent of the reductase activity of resistant rats was inhibited in incubations containing 35 μM warfarin.

The results of experiments designed to estimate the concentration of warfarin in liver during inhibition of prothrombin formation are shown in Table 2. Concentrations of warfarin were estimated assuming liver to be 70% water, wet weight. In control rats fed Purina Laboratory Chow, 25 μg of warfarin/100 g body weight inhibited prothrombin synthesis for at least 8 hr resulting in a prothrombin concentration of 53 per cent of normal.* The apparent concentration of warfarin in liver at that time was 3·6 μM. After 24 hr, the concentration of warfarin had fallen to 2·4 μM and the synthesis of clotting protein had been restored, at least partially, since the concentration of plasma prothrombin was 28 per cent of normal. Full inhibition for 24 hr requires that prothrombin concentration drop to about 12 per cent of normal. In control rats fed an excessive amount of vitamin K, 25 μg of warfarin did not inhibit prothrombin synthesis, but the concentration of warfarin in liver was approximately the same. In resistant rats, 10 mg of warfarin/100 g body weight blocked prothrombin synthesis for 24 hr (prothrombin, 12 per cent of normal) and the concentration of warfarin in liver at that time was 104 μM. This concentration of warfarin was probably marginal for full inhibition of prothrombin synthesis in resistant rats, since in a pilot experiment the injection of 10 mg of warfarin under similar conditions resulted in incomplete inhibition of prothrombin synthesis after 24 hr (prothrombin, 48 per cent of normal). The ineffectual dose of 1 mg of warfarin to these animals resulted, after 8 hr, in a warfarin concentration in liver of 80 μM.

The association of warfarin with subcellular fractions of liver from both control and resistant rats is shown in Table 3. The drug was bound to all membranes examined.

* The half-life of plasma prothrombin in rats given warfarin is approximately 8 hr.[14]

TABLE 2. PLASMA PROTHROMBIN AND THE CONCENTRATION OF WARFARIN IN LIVER 8 AND 24 HR AFTER THE INJECTION OF ^{14}C-WARFARIN IN CONTROL AND WARFARIN-RESISTANT RATS

		8 Hr			24 Hr		
Rats	Administered ^{14}C-warfarin (per 100 g body wt)	Prothrombin (% of normal)	^{14}C in liver (%)	Warfarin in liver* (μM)	Prothrombin (% of normal)	^{14}C in liver (%)	Warfarin in liver* (μM)
Control	25 μg	53	11·8	3·6	28	8·2	2·4
Control†	25 μg	87	12·6	4·5	80	7·4	2·7
Resistant†	10 mg	55	3·1	442·0	12	0·7	104·0
Resistant†	1 mg	90	5·4	80·0			

† Fed purified diet containing 10 μg K_1/g.
* Concentration of warfarin calculated assuming liver to be 70% water wet weight.

TABLE 3. RELATIVE SPECIFIC ACTIVITY OF ^{14}C-WARFARIN IN LIVER SUBCELLULAR FRACTIONS 8 AND 24 HR AFTER THE INJECTION OF ^{14}C-WARFARIN IN CONTROL AND WARFARIN-RESISTANT RATS*

Rats	Administered ^{14}C-warfarin (per 100 g body wt)	8 Hr					24 Hr				
		Mit	Mic	SM	RM	Cyt	Mit	Mic	SM	RM	Cyt
Control	25 μg	1·3	2·9	2·7	2·8	0·8	1·2	3·3	3·2	3·3	0·3
Control†	25 μg	1·2	3·0	3·0	2·8	0·6	1·4	4·2	3·5	4·3	0·3
Resistant†	10 mg	0·8	0·5	0·8	0·3	1·8	1·2	0·7	1·2	0·7	1·7
Resistant†	1 mg	0·6	0·6	0·7	0·4	1·7					

* Mit, mitochondrial fraction; Mic, microsomal fraction; SM and RM, smooth and rough membrane fraction, respectively; Cyt, cytosol. Relative specific activity is expressed as dis./min of ^{14}C-warfarin/mg protein; whole homogenate = 1·0.

† Fed purified diet containing 10 μg K_1 g.

The large amount of warfarin given to resistant animals resulted in a lower relative concentration of the anticoagulant in membrane fractions and an increase in the relative specific activity of cytosol. The amount of warfarin present in the livers of resistant rats was from 10–100 times higher than in control rats (see Table 2).*

The data in Table 3 show little or no difference in the distribution of warfarin in control rats given an excessive amount of vitamin K.

DISCUSSION

Reduction of vitamin K epoxide has been observed with several preparations of rat liver.[3,4] All have required the addition of DTT or dithioerythritol (DTE) for maximum activity. Crude systems, whole homogenates or the preparation used in these studies, convert 10–25 per cent of added substrate to the vitamin without DTT and are usually inactive beyond 30 min of incubation time. Purified microsomes are inactive unless a thiol reagent is added. Freshly prepared cytosol does not generate reductase activity with purified microsomes. These data suggest that DTT and DTE are artificial donors of reducing equivalents for the reaction. Both crude systems and purified microsomes from control rats convert from 40 to 70 per cent of added substrate to the vitamin if DTT or DTE is added. The activity of the reductase has not been linear with respect to protein concentration under any conditions tested.

The studies reported here and elsewhere[4,16] indicate that the reduction of vitamin K epoxide in resistant rats is less sensitive to inhibition by warfarin than the reaction in control rats. The difference between the liver reductase from control and resistant rats studied here was most apparent in the absence of added sulfhydryl but was also seen in the presence of DTT (see Fig. 1). These results indicate that the difference between the control and resistant enzyme is probably in reduction of the epoxide or transfer of reducing equivalents and not in systems which may normally provide reducing equivalents for the reaction. The data suggest that warfarin inhibits the transfer of reducing equivalents, since DTT partially reverses the inhibition.

* The lower relative specific activity of warfarin in microsomal membranes from resistant rats may also be due to decreased binding of warfarin in the liver of these animals as already observed by Lorusso and Suttie.[15]

Bell *et al.*[1] and Bell and Caldwell[16] have made a systematic study of the relationship between the ratio of epoxide to vitamin K in liver and the inhibition of clotting protein synthesis. In the present study, we have made a similar comparison, i.e. between the concentration of warfarin required to inhibit the reductase *in vitro* and the concentration of warfarin occurring *in vivo* during inhibition of prothrombin synthesis. This comparison is summarized in Table 4. In control animals, the concentration of warfarin *in vivo* was approximately six times that found necessary to

TABLE 4. SUMMARY OF THE CONCENTRATIONS OF WARFARIN REQUIRED TO INHIBIT VITAMIN K EPOXIDE REDUCTASE *in vitro* AND PROTHROMBIN FORMATION *in vivo* IN CONTROL AND WARFARIN-RESISTANT RATS

*In vitro**		*In vivo*†	
Control (μM)	Resistant (μM)	Control (μM)	Resistant (μM)
0·5	35	3	104

* Concentration of warfarin required to produce 90 per cent inhibition of reductase activity in incubations without the addition of DTT.
† Minimum concentration of warfarin observed in liver during inhibition of prothrombin formation.

inhibit the reductase *in vitro*, but this difference may be due in part to competitive affinity for warfarin by subcellular membranes, since the concentration of competing membrane protein for available warfarin was lower *in vitro* than *in vivo*. In resistant rats, where the concentration of warfarin was higher than in control rats, the difference between concentrations *in vitro* and *in vivo* was about 3-fold.

The comparisons shown in Table 4 are based on data *in vitro* from incubations without added DTT. In the presence of DTT (see Fig. 1), inhibition of the reductase required higher concentrations of warfarin and the difference in inhibitory concentrations of warfarin between control and resistant rats was less. At 2 mM DTT, reductase activity was not fully inhibited at any concentration of warfarin tested. Further experiments would be necessary, but correspondence between data *in vitro* and *in vivo*, as shown in Table 4, might be better if inhibition of the reductase were examined in incubations containing a low concentration of DTT, below 0·5 mM.

The biochemical basis for warfarin resistance proposed here is based on a new view of the mechanism of action of warfarin which invokes inhibition of vitamin K by vitamin K epoxide.[2] If this view is correct, decreased inhibition of vitamin K epoxide reductase by warfarin in liver from warfarin-resistant rats presents clear evidence of the biochemistry underlying resistance to the anticoagulant. Earlier observations by Lorusso and Suttie[15] that liver microsomes of resistant rats bind little warfarin may be interpreted from this study to be due, at least in part, to decreased affinity of the reductase for the inhibitory drug.

A further point may be made with regard to the role of the reductase and its inhibition by warfarin. Although we view the reductase as a regenerative enzyme (for regenerating vitamin K), it is unlikely that inhibition of the reductase by warfarin diminishes vitamin K to normally ineffectual levels. Thus, we have seen the importance of the enzyme not so much in terms of regeneration of vitamin K as in terms

of preventing accumulation of epoxide. From present evidence, however, regeneration of vitamin K may also be important if cyclic interconversion of the vitamin and the epoxide is necessary to the function of the vitamin.

The role of DT-diaphorase in reduction of vitamin K epoxide as propsed by Ernster *et al.*[6] remains to be studied. They reported that the activity of this enzyme was lower than normal in the liver of homozygous warfarin-resistant male rats. Later, they observed that the variation in DT-diaphorase activity between resistant and control rats resulted from a difference between laboratory strains and was independent of the resistant trait (personal communication). DT-diaphorase is a flavoprotein enzyme which catalyzes the oxidation of NADH and NADPH by naphthoquinones and several other oxidizing dyes. The enzyme is inhibited by oral anticoagulants and not inhibited less in resistant animals.[6] Decreased inhibition of the reductase by warfarin in resistant rats, observed in the present study and elsewhere,[16] does not exclude the possible role of DT-diaphorase in reduction of the epoxide, but an adequate explanation of the role of this enzyme must await further study and fuller characterization of the reductase.

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