Biochemical Pharmacology, Vol. 36, No. 17, pp. 2753–2757, 1987.
Printed in Great Britain.

0006-2952/87 $3.00 + 0.00
Pergamon Journals Ltd.

WARFARIN RESISTANCE

VITAMIN K EPOXIDE REDUCTASE OF SCOTTISH RESISTANCE GENES IS NOT IRREVERSIBLY BLOCKED BY WARFARIN

H. H. W. THIJSSEN
Dept. of Pharmacology, University of Limburg, P.O. Box 616, 6200 MD Maastricht, The Netherlands

(*Received* 28 *November* 1980; *accepted* 22 *February* 1987)

Abstract—The dithiothreitol-dependent vitamin K 2,3 epoxide (vitamin KO) reductase activity in liver microsomes of Scottish-derived warfarin-resistant Wistar rats (Tolworth Laboratory) was compared to that of susceptible Wistar rats. Under the test conditions, reductase activities in liver homogenates and in liver microsomes were comparable for both strains. The *in vitro* i50 of *S*-warfarin for microsomal reductase activity was 1 to 2 μM in both strains. The effect of *in vivo S*-warfarin was investigated after single doses, i.e. 0.2 and 1 mg/kg for the susceptible rats, and 1 and 5 mg/kg for the resistant rats. At 20 hr following the warfarin administrations in the susceptible strain, microsomal reductase was suppressed to about 30% of control. Microsomal reductase activity in the resistant strain was not reduced. Tissue and microsomal warfarin concentrations, however, were comparable in both strains. Wash experiments with microsomes which were treated *in vitro* with *S*-warfarin showed that vitamin KO reductase of the warfarin-resistant strain was not irreversibly inactivated by warfarin. The reactivation was mediated by DTT. The results suggest the following characteristic of the vitamin KO reductase of the Scottish resistance gene: contrary to the "normal" enzyme, the tight complex between the inhibitor and the resistant enzyme is liable to reactivation by reduction of the disulfide bridge in the active centre of the enzyme. This property explains the resistance for 4-hydroxycoumarin anticlotting activity.

The anticlotting activity of warfarin and other 4-hydroxycoumarin derivatives is imputed to the (irreversible) inhibition of the enzyme vitamin KO* reductase which forms an integral part of the vitamin K-cycle in the endoplasmatic reticulum of the hepatocyte. Interruption of this cycle attenuates the vitamin K-dependent "activation" of the clotting factors II, VII, IX and X. This vitamin K-dependent reaction contains the carboxylation of glutamic acid residues to gamma-carboxy-glutamic acids thereby creating calcium binding properties [1–4]. Beside its use in human therapy as an oral anticoagulant, warfarin is employed world wide as a rodenticide. This use has led to the selection of warfarin-resistant wild rat strains in some European countries and in the U.S.A. Studies on the vitamin K-dependent microsomal systems, i.e. vitamin K and vitamin KO reductase, of warfarin-resistant rats thus far showed that (a) total vitamin KO reductase activity in liver microsomes is lower [5]; (b) the *in vitro* sensitivity of vitamin K and vitamin KO reductase for warfarin inhibition is reduced [1, 5–7]; (c) the *in vitro* incubation of microsomal vitamin KO reductase with vitamin KO leads to the formation of 2(3)-hydroxylated vitamin K [8, 9].

Recently, however, MacNicoll showed that vitamin KO reductase of warfarin-resistant Wistar-derived rats originating from resistant Scottish wild rats is similar to that of susceptible rats in substrate and inhibitor (warfarin) affinity [10]. This Scottish derived strain was introduced into the laboratory animals service department of our faculty before these features were known. In the present study, we report on additional characteristics of the Scottish derived vitamin KO reductase that may explain the warfarin resistance of this strain.

* Abbreviations used: vitamin K: vitamin K1; vitamin KO: vitamin K1 2,3 epoxide; DTT: dithiothreitol.

MATERIALS AND METHODS

The Scottish derived warfarin-resistant strain, interbred with a Wistar strain, was obtained from Dr Greaves (Agricultural Science Service, Tolworth Laboratory, Tolworth, Surbiton, Surrey, U.K.) and is bred at our laboratory animals service department. The rats were fed standard diet (Hope Farms, Woerden, The Netherlands) and tap water *ad libitum*. Male animals (250–300 g) were used for the experiments. Warfarin susceptible male Wistar rats (250–300 g) were obtained from Winkelman (Borchen, F.R.G.)

Materials

Vitamin KO was prepared from vitamin K (Merck, Darmstadt, F.R.G.) following the method by Tishler [11]. *S*-warfarin was prepared from racemic warfarin (Sigma Chemicals) by the method of West *et al.* [12]. All other chemicals were of analytical grade and were obtained from regular suppliers.

Methods

Pharmacokinetic studies. The pharmacokinetics of *S*-warfarin were studied following a single subcutaneous dose (0.4 mg/kg). Free access to blood of the rats was obtained via a permanent femoral artery catheter as described previously [13]. Blood (200 μl)

was sampled at regular time intervals for drug assay and clotting activity. Biological half-life, volume of distribution and total plasma clearance were estimated as described previously [13].

Microsomes of liver tissue were prepared from control animals and from animals which had been given a single dose of *S*-warfarin subcutaneously 20 hr before. Under ether anesthesia the abdomen of the rats was opened. Blood was withdrawn from the abdominal aorta and mixed immediately with 1/9th of its volume of 0.1 M trisodiumcitrate. The liver was perfused with ice-cold saline via the vena porta, then excised, rinsed and homogenized in 3 vol. (w/v) of ice-cold buffer (0.02 M Tris–HCl, 0.25 M sucrose, 0.15 M KCl, pH 7.4) using the Potter technique. The homogenates were centrifuged at 10,000 *g* for 30 min. Microsomes were pelleted from the 10,000 *g* supernatant by centrifugation at 100,000 *g* for 60 min. The microsomes were resuspended in the Tris buffer and repelleted. This procedure was repeated with a Tris–HCl buffer (0.02 M, pH 7.4) containing 1 M of NaCl. The final microsomal pellet was resuspended in the latter buffer to obtain a microsomal protein concentration of about 15 mg/ml. The protein content was measured by the Lowry method. The microsomes could be stored at −70° for over three months without losing activity.

Vitamin KO reductase assay. The assay procedure for vitamin KO reductase activity as described previously [14] was adapted for small size tissue samples. In 3 ml reaction vessels 0.02 ml of tissue (=liver) homogenate or microsomal preparation was mixed with 0.075 ml cold Tris–HCl buffer (0.02 M, pH 7.4). The mixture was preincubated for 3 min at 30°. DTT, 2 μl of a 0.1 M solution in Tris buffer, was added and the mixture was incubated for another 3 min. The substrate vitamin KO, 10 nmole in 2 μl of isopropanol, was added and the incubation was continued for 10 min. The reaction was stopped by the addition of 0.9 ml ice-cold isopropanol. To the mixture was added 0.5 ml of 0.01 M silver nitrate and 0.6 ml hexane containing 2.5 μg of tocophorolacetate (Sigma Chemicals) for internal standardization. (The addition of $AgNO_3$ to the mixture prevents the extraction of DTT and thereby the formation of vitamin K derived products on the column [14, 15].) The mixtures were centrifuged following brief, 2 × 10 sec, vortex mixing. The hexane phase was washed with 0.5 ml 1% sodium chloride and then it was evaporated to dryness under a stream of nitrogen at 35°. The residue of the hexane phase was redissolved immediately in 0.05 ml of iso-propanol and analyzed by HPLC as described previously [14]. All reductase assays were performed in duplo.

Miscellaneous. Warfarin content in plasma, liver homogenates, and liver microsomes were assayed as described previously [16]. Blood clotting activity was assayed by the thrombotest [13].

The results are presented as mean ± SD. Differences between results were compared using the Student's *t*-test.

RESULTS

The warfarin resistance of the rats bred at our faculty was ascertained by adding warfarin to the drinking water. Normal Wistar rats did not survive 3 mg racemic warfarin per liter drinking water for 2 weeks whereas no casualties were observed for the resistant rats even when the warfarin dosing was five times as high. The pharmacokinetics of *S*-warfarin following single dose experiments are summarized in Table 1. Slight but significant differences between the strains were observed for the distribution volume and for the plasma clearance. The clotting activity, 24 hr after dosing, was much more depressed in the susceptible rat. The rates of decline of the plasma clotting activity (PCA), however, were comparable (Table 1).

Table 1. Comparative pharmacokinetics and pharmacodynamics of a single dose of *S*-warfarin (0.4 mg/kg s.c.) in Wistar rats and warfarin-resistant rats[a]

	Wistar (N = 6)	Resistant strain (N = 3)
t1/2 (hr)	12.5 ± 2	15 ± 1
Vd (ml/kg)	115 ± 14	91.6 ± 5*
Cl (ml/hr.kg)	6.6 ± 1.3	4.2 ± 0.3*
k^{deg}_{PCA} (h^{-1})[b]	3.8 ± 0.2	4.5 ± 2 (n = 5)
PCA (% of normal)[c]	12 ± 2.6	57 ± 4

[a] Pharmacokinetic constants are estimated via the area under the curve method [13]. The data are based on plasma warfarin concentrations. The results are mean ± SD. $t_{\frac{1}{2}}$ = half-life; V_d = volume of distribution; Cl = total plasma clearance; * $P < 0.05$.

[b] Degradation rate of plasma clotting activity (PCA). The constant was derived from the initial decline curve (0–8 hr) of the clotting activity [13]

[c] Plasma clotting activity as % of normal 24 hr following the drug administration.

Microsomal vitamin KO reductase activities in the resistant strain and the susceptible Wistar strain were comparable, 31 ± 4 (N = 6) vs 36.5 ± 8 (N = 6) pmole vitamin K formed per mg microsomal protein per min. The warfarin sensitivity of the microsomal vitamin KO reductase is shown in Fig. 1. The i50 of *S*-warfarin was about 2 μM for both reductases. The reductase activities in liver homogenates and liver

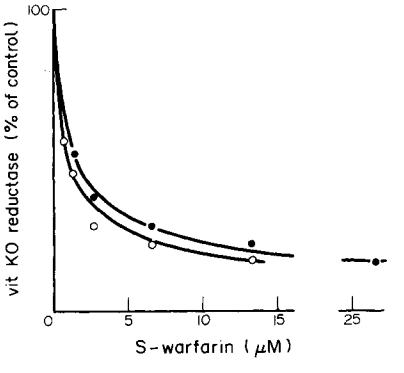


Fig. 1. The effect of *S*-warfarin on the vitamin KO reductase activity in liver microsomes of susceptible (○) and resistant (●) rats. The reaction mixtures were pre-incubated in the presence of *S*-warfarin for 5 min whereafter DTT was added (see Materials and Methods).

Table 2. The effect of single dose *S*-warfarin on vitamin KO reductase activity in liver tissue homogenate and liver microsomes of normal and resistant rats[a]

Rats	S-warfarin (mg/kg)	Vitamin KO reductase: Liver homogenate, vit K ng/g tissue·min	Vitamin KO reductase: Liver microsomes, vit K ng/mg protein·min
Normal	0	810 ± 140 (N = 6)	15 ± 2 (N = 6)
	0.2	280 ± 20 (N = 3)*	6 ± 1 (N = 3)*
	1	248 ± 30 (N = 3)*	4 ± 0.5 (N = 3)*
Resistant	0	770 ± 40 (N = 6)	14 ± 0.5 (N = 6)
	1	700 ± 10 (N = 3)*	14 ± 5 (N = 3)
	5	520 ± 20 (N = 3)*	11.5 ± 2 (N = 3)*

[a] S-warfarin was administered subcutaneously 20 hr before. Control rats received saline. The rats were sacrificed 20 hr later. The results are given as mean ± SD.
* $P < 0.05$ or better when compared to control.

microsomes of normal and resistant rats 20 hr following single doses of *S*-warfarin are shown in Table 2. In susceptible rats, following 0.2 or 1 mg/kg *S*-warfarin reductase activity in liver homogenates and liver microsomes was reduced by about 70%. The resistant rats on the contrary, showed only slight reductions of the reductase activity; 10 and 35% in liver homogenates for 1 and 5 mg/kg, respectively. About 20% reduction was observed in microsomes for the 5 mg/kg dose.

To see whether differences in warfarin distribution could account for these observations, the warfarin levels in the various tissues were assayed (Table 3). As can be seen, no gross differences between the susceptible and resistant strain were observed in warfarin distribution.

One of the characteristics of the warfarin effect on *in vitro* vitamin KO reductase activity is the apparent irreversibility of the inhibitory action [3, 14, 17]. This phenomenon was tested for the resistant and susceptible strains. In Scheme 1, the experimental design is presented. The initial *S*-warfarin concentrations used for pre-incubation of the microsomes were 1 and 10 μg per ml of microsomal suspension. The results are summarized in Table 4. The reductase activity following a pre-incubation with warfarin for 20 min at room temperature was strongly reduced in microsomes of susceptible rats, 70 and 80% inhibition for 1 and 10 μg/ml *S*-warfarin concentrations. (It should be noted that if warfarin was available for dilution, its final concentration in the reductase assay would be five times less; see reductase assay in Methods section.) Reductase activity in the pre-incubated microsomes of resistant rats was reduced by 20 and 60%. Following the washing procedure, reductase activity in resistant microsomes recovered to 90 and 75% of control activity for 1 and 10 μg/ml *S*-warfarin treated microsomes. Microsomes of susceptible rats showed hardly any reactivation. However, there was no difference between susceptible and resistant microsomes in the amount of warfarin they retained; about 350 and 800 ng/ml microsomal suspension for 1 and 10 μg/ml *S*-warfarin pre-incubated microsomes (Table 4). Our own observations (unpublished) showed that in susceptible microsomes warfarin inhibition reduced in time in the presence of high (50 mM) DTT concentrations. Therefore, to get a clue on the discrepancy between reductase activity and microsomal warfarin content in resistant microsomes, the 10 μg/ml *S*-warfarin treated microsomes were also washed with buffer containing 2 mM DTT. Following this procedure recovery of reductase activity was 100% in resistant microsomes, whereas in susceptible

Table 3. Tissue warfarin distribution in normal and resistant rats 20 hr following an acute subcutaneous *S*-warfarin dose

Rats (N = 3)	*S*-warfarin (mg/kg)	Tissue content[a]: Plasma	Liver	Microsomes
Normal	0.2	0.16 ± 0.03	0.28 ± 0.01	11.7 ± 3
	1	1.20 ± 0.07	1.15 ± 0.1	15.3 ± 2.4
Resistant	1	1.32 ± 0.13	0.82 ± 0.07	9.2 ± 1.1
	5	5.30 ± 0.8	2.00 ± 0.34	14.3 ± 1

[a] Concentration (mean ± SD) in plasma and liver, μg per gram wet tissue weight; in microsomes, ng per mg protein content.

Table 4. The effect of wash procedures on vitamin KO reductase activity and on warfarin content in microsomes of normal (N) and resistant (R) rats[a]

		Reductase activity[c]		Warfarin content[d]	
	Warf.[b]	N	R	N	R
Pre-incubated microsomes	0	100	100		
	1	31	78		
	10	19	38		
Washed microsomes	0	100	100		
	1	36	88	320 (1)	360 (1.2)
	10	25	76	700 (2.3)	900 (2.9)
DTT-washed microsomes[e]	0	100	100		
	10	29	100	250 (0.8)	45 (0.15)

[a] The treatment of the microsomes was as outlined in Scheme 1.

[b] S-warfarin was added in the pre-incubation phase in 1 and 10 μg amounts per ml (3.25 and 32.5 ±M, respectively) of microsomal suspension.

[c] Reductase activity is presented as percentage of the control activity, i.e. the activity in the absence of *S*-warfarin. Reductase assays were in duplo.

[d] Warfarin (ng/ml) recovered in microsomes. The number in parenthesis represents μM.

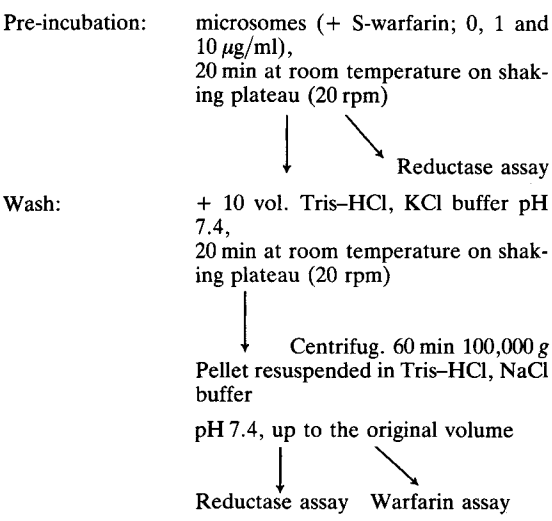
[e] The microsomes were washed with buffer containing 2 mM of DTT.

microsomes only a slight reactivation was observed. In comparison to the buffer washed microsomes, the warfarin content in the DTT treated microsomes was reduced about 3-fold in the susceptible, and about 20-fold in the resistant microsomes (Table 4).

DISCUSSION

In a recent study, MacNicoll [10] showed that warfarin resistance is not defined by merely one set of biochemical parameters, i.e. warfarin resistance originating from a Scottish wild strain appeared to differ from that of a Welsh strain. All studies up to then were performed with rats derived from Welsh wild type resistant rats and the picture that emerged was straightforward [5–7]: the target for warfarin action, i.e. vitamin KO reductase, differs in the way that its susceptibility to warfarin is strongly reduced. Whether at the same time the total vitamin KO reductase activity is reduced can be questioned. MacNicoll made it plausible that due to the high substrate K_m of the Welsh type resistant vitamin KO reductase, the assayed reductase activity was low in comparison with susceptible rats [10].

The present results affirm the observation of MacNicoll; vitamin KO reductase of the Scottish-derived warfarin-resistant strain is not altered with respect to the *in vitro* susceptibility to warfarin nor is the liver reductase activity at the test conditions different from normal rats. The i_{50} of *S*-warfarin observed in the present study for the *in vitro* vitamin KO reductase reaction, i.e. 1–2 μM, is comparable to previous results observed in our laboratory [14] and to the data of others [1, 5–7, 10].



Scheme 1. Outline of the *in vitro* *S*-warfarin treatment and subsequent wash procedure of microsomes.

As there was no difference between the susceptible and the resistant strain in warfarin distribution, i.e. the amounts of warfarin present in liver tissue and liver microsomes after a single dose were comparable (Table 3), warfarin resistance in the Scottish derived strain apparently is neither a matter of pharmacokinetic differences.

Rather puzzling were the observations that the warfarin contents in resistant microsomes following various routes of warfarin administration, i.e. *in vivo* and *in vitro*, were comparable to those of the susceptible strain (Tables 3 and 4), yet their reductase activity was not or only slightly reduced. The solution to this problem was given by the experiments that investigated whether or not the warfarin effect is irreversible (Table 4). The irreversibility of the inhibition by 4-hydroxycoumarins has been stressed by several authors and is thought to be fundamental for the mechanism of interaction [3, 14, 19]. In the washing experiments, it appeared that DTT (2 mM) was able to "displace" warfarin of its microsomal binding site(s) (vitamin KO reductase?). This displacement was much more effective (7-fold) in microsomes of resistant rats and resulted in complete recovery of the reductase activity of the resistant microsomes. We now can explain why the reductase activity of resistant microsomes containing for instance 900 ng of *S*-warfarin/ml (*ca* 3 μM) was only reduced by about 30%, whereas that of susceptible microsomes was reduced by 70% (Table 4). Namely, at the reaction conditions of the assay (i.e. 2 mM DTT, 30°), warfarin is displaced of the resistant vitamin KO reductase and is diluted 5-fold in the reaction mixture. This also explains why, contrary to the findings of MacNicoll (Table III in the paper of MacNicoll; ref. 10), in the present study only weak effects on the microsomal KO reductase activity were observed following the *in vivo* warfarin administration (Table 2). Whereas MacNicoll used the 100,000 *g* pellet as such, in the present study the microsomes were routinely washed twice during their isolation (see Methods section), removing the greater part of warfarin. The "irre-

versibly" bound warfarin in the resistant microsomes is displaced during the assay.

Fasco *et al.* [18] proposed the following mechanism to explain the irreversible inhibition of vitamin KO reductase by 4-hydroxycoumarins: vitamin KO reductase is oxidized (disulfide-bond formation) during the reduction of vitamin KO. The oxidized enzyme is transferred to the reduced form (2 sulfhydryl groups) by an electron donor (*in vitro*: DTT). The oral anticoagulants interact with the oxidized enzyme to form a stable complex and preventing reactivation (reduction of the disulfide bond) of the enzyme. Silverman [19, 20], on the other hand, postulated bond formation between the reductase and the 4-hydroxycoumarins via one of the SH groups of the active site of the enzyme. Reactivation might occur through (base catalyzed) substitution of the bound 4-hydroxycoumarin via the second SH group.

The present results as well as observations not published, i.e. reduced or no *in vitro* inhibition occurs if warfarin is added to reaction mixtures after the microsomes are pre-incubated with DTT (see also, ref. 18), decide in favour of the Fasco-model. According to this model the warfarin resistance in the Scottish strain should be seen as follows: the vitamin KO reductase is altered in such a way that the S–S bond of the oxidized enzyme, notwithstanding the enzyme being tightly complexed with the inhibitor, is accessible for the reductor (DTT) to become reduced. The enzyme in its reduced form has no or only weak affinity for the 4-hydroxycoumarins. The inhibitor diffuses out of the "vitamin K compartment" and is diluted by elimination. This mechanism of resistance once again stresses the importance of the "irreversible" inhibition of vitamin KO reductase for the oral anticoagulants to function.

In summary, the results unequivocally show that warfarin resistance in rats is based on at least two mechanisms: (1) the Welsh type resistance is due to a vitamin KO reductase with reduced substrate affinity and reduced affinity for 4-hydroxycoumarins. The enzyme also shows lowered affinity for the transition state intermediate 3-hydroxylated vitamin K; (2) The Scottish type resistance is based on a vitamin KO reductase which — although sensitive to 4-hydroxycoumarins — is not irreversibly inactivated by these compounds.

REFERENCES

1. D. S. Whitlon, J. A. Sadowski and J. W. Suttie, *Biochem.* **17**, 1371 (1978).
2. J. W. Suttie, *Fedn. Proc. Fedn. Am. Socs. exp. Biol.* **39**, 2730 (1980).
3. M. J. Fasco and L. M. Principe, *J. biol. Chem.* **257**, 4894 (1982).
4. C. Vermeer, *Molec. Cell. Biochem.* **61**, 18 (1984).
5. E. F. Hildebrandt and J. W. Suttie, *Biochemistry* **21**, 2406 (1982).
6. A. Zimmermann and J. T. Matschiner, *Biochem. Pharmac.* **23**, 1033 (1974).
7. M. J. Fasco, E. F. Hildebrandt and J. W. Suttie, *J. biol. Chem.* **257**, 11210 (1982).
8. M. J. Fasco, P. C. Preusch, E. Hildebrandt and J. W. Suttie, *J. biol. Chem.* **258**, 4372 (1983).
9. P. C. Preusch and J. W. Suttie, *J. Nutr.* **114**, 902 (1984).
10. A. D. MacNicoll, *Biochim. biophys. Acta* **840**, 13 (1985).
11. M. Tishler, L. F. Fieser and N. L. Wendler, *J. Am. chem. Soc.* **62**, 2866 (1940).
12. B. D. West, S. Preis, C. H. Schroder and K. P. Link, *J. Am. chem. Soc.* **83**, 2676 (1961).
13. H. H. W. Thijssen, L. G. Baars and M. J. Reijnders, *J. Pharm. Pharmac.* **35**, 793 (1983).
14. H. H. W. Thijssen, C. A. T. Janssen and M. J. Drittij-Reijnders, *Biochem. Pharmac.* **35**, 3277 (1986).
15. E. F. Hildebrandt, P. C. Preusch, J. L. Patterson and J. W. Suttie, *Archs Biochem. Biophys.* **228**, 480 (1984).
16. H. H. W. Thijssen, L. G. Baars and M. J. Reijnders, *J. Chromatogr.* **274**, 231 (1983).
17. R. Wallin, S. D. Patrick and J. O. Ballard, *Thromb. Haemostas.* **55**, 235 (1986).
18. M. J. Fasco, L. M. Principe, W. A. Walsh and P. A. Friedman, *Biochemistry* **22**, 5655 (1983).
19. R. B. Silverman, *J. Am. chem. Soc.* **102**, 5421 (1980).
20. R. B. Silverman, *J. Am. chem. Soc.* **103**, 5939 (1981).