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THE EFFECT OF *S*-WARFARIN ADMINISTRATION ON VITAMIN K 2,3-EPOXIDE REDUCTASE ACTIVITY IN LIVER, KIDNEY AND TESTIS OF THE RAT

H. H. W. THIJSSEN, C. A. T. JANSSEN and M. J. DRITTIJ-REIJNDERS
University of Limburg, Department of Pharmacology, P.O. Box 616, 6200 MD Maastricht, The Netherlands

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Abstract—The dithiothreitol-dependent vitamin K 2,3-epoxide (vitamin KO) reductase activity was assayed in rat liver, kidney and testis microsomes. Rat kidney and testis showed vitamin KO reductase activity. The activity was about one tenth of the activity present in liver microsomes. The effect of *in vivo* *S*-warfarin was investigated after single doses, i.e. 0.2, 0.4 and 1 mg/kg, and after its chronic administration, i.e. 4.8 μg/kg/hr for 3 days. At 20 hr following the acute warfarin administration vitamin KO reductase in liver microsomes was depressed in a dose-dependent way, 50, 30 and 20% of control activity. Vitamin KO reductase in testis was not affected, and in kidney reductase activity was only reduced after the highest warfarin dose, 40% of control activity. Following chronic administration of warfarin, vitamin KO reductase activity was reduced in liver as well as in kidney and testis microsomes, 15–20, 40 and 60% of control activity in liver, kidney and testis, respectively. Blood clotting activity was about 14% of normal (thrombotest). Vitamin KO reductase activity in tissue microsomes was inhibited by warfarin added *in vitro*. Tissue and microsomal warfarin concentration were assayed. Following the acute administration, warfarin was poorly distributed into kidney and testis. Following the chronic administration, warfarin tissue to plasma ratio was about 3 for liver, but 0.5 for kidney and testis. The results indicate that during chronic therapy with oral anticoagulants vitamin K-dependent systems in non-hepatic tissues are reduced. However, this reduction is less than the reduction of the hepatic system. This is determined mainly by the pharmacokinetic behaviour of the 4-hydroxycoumarins.

In the last decade considerable progress has been made in the understanding of the biochemical function of vitamin K. In the endoplasmatic reticulum of the hepatocyte, the hydroquinone of vitamin K functions as a cofactor for a carboxylase which converts glutamic acid residues in substrate proteins to gamma-carboxyglutamic acids, thereby creating calcium interacting properties. Concomitant with the carboxylation, vitamin KO* is generated. By specific reductases (DTT-dependent vitamin KO reductase, DTT- and NADH-dependent vitamin K reductases) vitamin KO is converted to vitamin K and vitamin K in its turn to the hydroquinone. The oral anticoagulants have been shown to inhibit the microsomal DTT-dependent reductase reactions. As the microsomal vitamin K reduction can be mediated by a warfarin insensitive NADH-dependent pathway it is suggested that the anticlotting activity of the oral anticoagulants depends chiefly on the inhibition of the vitamin KO reductase [1–5].

It is established nowadays that microsomal vitamin K-dependent carboxylases also are present in tissues outside the liver, such as lung, bone, kidney, placenta, testis, etc. [6–13] and that tissue-specific gamma-carboxyglutamic acid containing proteins, exist. Their physiological functions, however, are still unknown [11, 14]. Oral anticoagulants have been in clinical use for more than 40 years. Up to now no indications have ever been presented suggesting that oral anticoagulants might affect, for instance, renal or testicular function [15]. The following factors might underly this given: (1) the target enzyme vitamin KO reductase is not present in these non-hepatic tissues; (2) the target enzyme in these tissues is less sensitive; (3) the drugs do not reach effective concentrations in these tissues; (4) the tissue vitamin K-dependent proteins are not as essential as to induce noticeable organ insufficiencies [11].

To get a clue on the mechanisms involved, we investigated vitamin KO reductase activity in kidney and testis of the rat, and the effect of acute and chronic warfarin administration thereupon.

MATERIALS AND METHODS

Male Wistar rats (300–350 g) were used. The animals had free access to food and drinking water. *S*-warfarin was prepared from racemic warfarin (Sigma Chemicals) by the method of West *et al.* [16]. Vitamin KO was prepared from vitamin K (Merch, Darmstadt, Federal Republic Germany) by the Tishler method [17].

Acute studies. Rats were given *S*-warfarin, soluted in 0.1 M potassium phosphate buffer pH 7.4, as a single dose subcutaneously. The doses were 0.2, 0.4 and 1 mg/kg (N = 3). Control rats (N = 6) received saline subcutaneously. Twenty hours later the animals were sacrificed and the organs removed for drug analysis and preparation of tissue microsomes (see later).

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

Chronic studies. Rats (N = 4) were given *S*-warfarin chronically for 3 days by means of a subcutaneously implanted osmotic minipump (Alzet®). The dose was 4.8 μg/hr/kg. Control rats (N = 4) obtained saline via the pump. At the established time, the rats were sacrificed and tissues were removed. This experiment was performed twice.

Microsomes. Under ether anesthesia, the abdomen and thorax of the rats were opened. Blood was withdrawn from the vena cava superior and mixed immediately with 1/9th of its volume of 0.1 M trisodium citrate. Via the abdominal aorta, the kidneys and the liver were perfused with ice-cold saline. The organs, i.e. liver, kidney and testes, were removed, rinsed and homogenized in 3 vol. (w/v) of ice-cold buffer (0.02 M Tris HCl, 0.15 M KCl, pH 7.4), using the potter technique. Kidneys and testes of rats from one experimental group were pooled. The homogenates were centrifuged at 10,000 *g* for 30 min. Microsomes were pelleted from the 10,000 *g* supernatants 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 (by chance, we found this extra wash procedure to enhance microsomal reductase activity about threefold). The microsomes were 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 were stored at −70°.

Vitamin KO reductase assay. The microsomal vitamin KO reductase activity was determined using DTT as the reducing agent [1, 18]. The enzymatic product, vitamin K, was assayed by an adapted HPLC method. The detailed procedures were as follows: 0.400 ml microsomal suspension (6–8 mg protein) were mixed with 0.59 ml of 0.02 M Tris–HCl buffer pH 7.4. The mixture was preincubated for 3 min at 30°. DTT, 10 μl of a 1 M solution, was then added and the mixture was incubated for another 2 min. The substrate vitamin KO, 0.1 μmole in 5 μl of iso-propranol, was added and the incubation continued. Incubation times were 15 min for liver and 45 min for kidney and testis. Incubations were performed in duplicate. The reaction was stopped by mixing a volume of the reaction mixture (0.4 ml) with 4 ml of iso-propanol/water (3:1), containing an internal standard, i.e. 25 μg of tocophorolacetate (Sigma Chemicals). The resulting mixture was extracted with 2 ml of hexane by shaking on a horizontal shaking table for 5 min. Following a 1 min centrifugation (2500 rpm) at 4°, the hexane phase was evaporated to dryness within 15 min under a stream of nitrogen at 35°. To suppress photochemical degradation of vitamin K, all procedures were done in yellowish light. The residue of the hexane phase was redissolved immediately in 0.1 ml of iso-propanol and analyzed by HPLC and u.v. absorption at 250 nm. The following system was used: analytical column, Lichrosorb 5RP18 (150 × 4.6 mm, Chrompack, The Netherlands); eluent, acetonitrile/water/dichloromethane (100/4/20, v/v/v); flow, 2 ml/min. The amount of vitamin K formed was determined from the peak height ratio vitamin K/internal standard and a calibration graph. Following every 10 sample runs 50 μl of DMSO was injected into the system to keep the column in good condition.

Miscellaneous. Warfarin content in plasma, tissue homogenates, and tissue microsomes were assayed by an HPLC method [19] with some modifications in the extraction procedure for the tissue homogenates and microsomes. Before extraction, homogenates and microsomes (to which acenocoumarol was added as internal standard) were mixed with equal volumes of acetonitrile and allowed to stand for 10 min. Then, twice the volume of buffer (phosphate/citrate, pH 4.2) was added and the coumarins were extracted [19]. Blood clotting activity was assayed by the Thrombotest as described previously [20].

RESULTS

Vitamin KO reductase assay

The use of the HPLC technique to follow vitamin KO reductase activity is common practice [18]. We

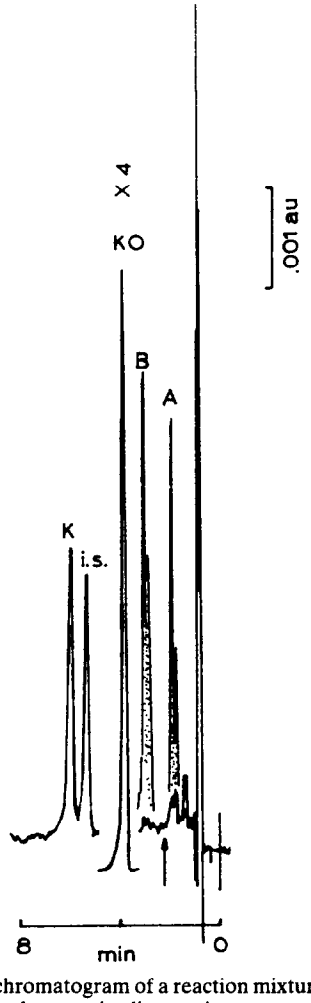

Fig. 1. HPLC-chromatogram of a reaction mixture assaying vitamin KO reductase in liver microsomes. Peak i.s. denotes internal standard. Dashed peaks refer to "spurious" vitamin KO derived products, A = DTT adduct, B = hydroxy vitamin K. Note double peaks (see text for further explanation). Arrow indicates place of vitamin K hydroquinone in the chromatogram.

adapted the system used normally by others to be able to use an internal standard (Fig. 1). Calibration showed a linear relationship between the peak height ratio of vitamin K and internal standard and the concentration of vitamin K (range 0.1–10 μg vitamin K/ml, $r = 0.998$). Hildebrandt *et al.* showed recently that the incubation of vitamin KO in the presence of DTT may lead to a vitamin KO–DTT adduct, and to hydroxy vitamin K. The formation of these products strongly depends on the conditions used during evaporation of the hexane phase [21]. At the assay conditions used in our experiments the formation of such products was rare. Reductase assays showing incidentally these products were not used in our results. It is noteworthy that—unlike reports in literature [21, 22]—in our system the products appeared as double peaks suggesting two compounds (isomer 2 and 3) at least (Fig. 1). The formation of the hydroquinone of vitamin K in microsomal vitamin KO reductase reactions has been proven by several authors [18, 21]. In our reductase experiments, however, we never observed a hydroquinone peak. Apparently the formed hydroquinone was converted back to vitamin K at the extraction conditions.

Vitamin KO reductase activity

Besides in liver, vitamin KO reductase activity was found to be present in kidney and testis microsomes. The *in vitro* reaction proceeded linearly for at least 20 min in liver microsomes and 90 min in kidney and testis (Fig. 2). The vitamin KO reductase activity per mg of microsomal protein of kidney and testis appeared to be about 1/10th of the activity in liver; 13 ± 4.4 (mean ± SD, N = 14) pmoles of vitamin K were formed in liver microsomes per min per mg of protein. For kidney and testis, the data were 1 ± 0.3 (N = 4) and 1.8 ± 0.2 (N = 4), respectively. Comparable to liver vitamin KO reductase, kidney and testis microsomes needed DTT to convert the substrate to vitamin K. Reducing agents such as NAD(P)H were without effect. Warfarin added *in vitro*, inhibited the reductase activities similarly (Fig. 3).

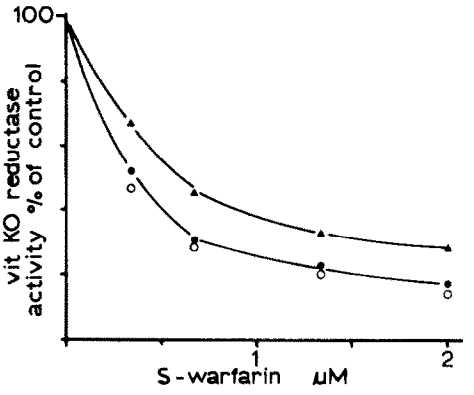

Fig. 3. The effect of *S*-warfarin on the vitamin KO reductase activity in liver (●), kidney (○), and testis (▲) microsomes. *S*-warfarin was added to the reaction mixtures before the pre-incubation period started (see Materials and Methods).

Acute warfarin experiments

The reductase activity in liver, kidney and testis, 20 hr after single doses of *S*-warfarin, is shown in Table 1. Reductase activity in liver microsomes decreased in a dose-dependent way; 50, 30 and 20% of control activity following 0.2, 0.4 and 1 mg/kg *S*-warfarin administration. Testis reductase activities in the warfarin groups were not different from control. Kidney reductase activity in the group of the highest warfarin dose was less, about 40% of control.

The warfarin concentrations observed in the different tissue compartments are presented in Table 2. Warfarin concentration in tissues increased with dose but not in a linear way, plasma concentrations increased more than linear. The warfarin con-

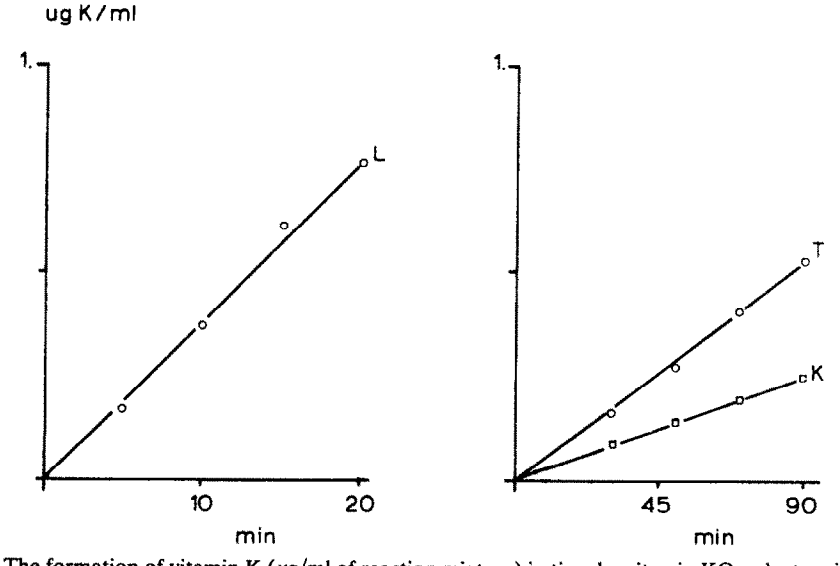

Fig. 2. The formation of vitamin K (μg/ml of reaction mixture) in time by vitamin KO reductase in liver (L), kidney (K) and testis (T) microsomes. Reaction conditions were as described in Materials and Methods.

Table 1. The effect of single dose *S*-warfarin on vitamin KO reductase activity in rat liver, kidney and testis microsomes*

Microsomes	pmole vitamin K/mg·min† Liver	Kidney	Testis
Control	16 ± 4 (N = 6)	1.3	1.6
+ warf 0.2 mg/kg	8 ± 2 (N = 3)	1.3	1.9
+ warf 0.4 mg/kg	5 ± 1 (N = 3)	1.2	1.5
+ warf 1 mg/kg	3 ± 1 (N = 3)	0.5	1.9

* *S*-warfarin was administered subcutaneously. Control rats received saline. The rats were sacrificed 20 hr later.

† Reductase activities of liver microsomes were from individual animals (mean ± SD), Reductase activities of kidney and testis microsomes were from pooled organs. Assays were performed *in duplo*. The activity is expressed as the amount vitamin K formed per mg microsomal protein per min.

Table 3. The effect of chronic *S*-warfarin administration on microsomal vitamin KO reductase activity*

	pmole vitamin K/mg·min Liver		Kidney		Testis	
Experiment	1	2	1	2	1	2
Control	10.4 ± 1.5	12.7 ± 1.5	0.8	0.7	1.6	1.8
Warfarin	2.3 ± 0.3	2.0 ± 0.6	0.3	0.3	1.1	1.0

* *S*-warfarin was administered chronically by means of the Alzet osmotic minipump. Reductase activity was measured in microsomes of the individual livers (mean ± SD, N = 4). The microsomes of kidney and testis were prepared from the pooled organs of 4 rats. Reductase assays were performed *in duplo*.

centrations in kidney and testis were about 3 times lower than in liver. Notwithstanding the extensive washings, warfarin was found in liver microsomes. In microsomes from kidney and testis, no warfarin was detected following the 0.2 and 0.4 mg/kg dose. Following the 1 mg/kg dose, the drug was observed in the microsomes of these tissues, albeit in faint amounts (Table 2). The blood clotting activity was about 40% of the control in the 0.2 mg/kg rats and about 15% in the 0.4 and 1 mg/kg rats.

Chronic warfarin studies

Experiments with constant rate infusion of *S*-warfarin in rats (via the osmotic minipump) learned that for the Wistar strain in our institute steady-state warfarin levels are reached on day 2 of infusion. Anticlotting activity, depending on the amount of warfarin infused, is maximally depressed at day 2 and remains fairly stable as long as the infusion lasts (unpublished results). The *S*-warfarin dose used in the present experiments (i.e. 4.8 μg/hr/kg) usually lowered blood clotting activity to about 10% of normal.

The effect of chronic warfarin administration on the vitamin KO reductase activity recovered from liver, kidney and testis microsomes is presented in Table 3. Reductase activities were less in the tissues, about 20, 45 and 60% of control values for liver, kidney and testis, respectively. The observed warfarin concentrations in tissue homogenates and tissue microsomes are given in Table 4. Liver warfarin concentrations were higher than plasma concentrations (liver tissue distribution about 3), kidney and testis concentrations were lower. Besides in liver, warfarin was also found to be present in kidney and testis microsomes. Blood clotting activity in the warfarin rats was 14 ± 8 (±SD, N = 8)% of control thrombotest value.

DISCUSSION

The existence of specific vitamin K-dependent proteins has been shown for various non-hepatic tissues. Their precise function is not known yet, be it that the vitamin K-dependent bone protein osteocalcin probably functions to prevent early calcification of the growing bone [22] and also to regulate bone resorption [23]. The typical skeletal malformations observed in offspring from mothers taking oral anticoagulants during pregnancy, the so-called warfarin syndrom, points in the same direction [24]. So far, this is the only indication that vitamin K-antagonists may affect functions other than the blood clotting system to evoke infirmities. The presence of the enzyme vitamin KO reductase, i.e. one of the target enzymes for coumarin attack, in extrahepatic tissues has not been shown before. In the present study we have shown the presence of vitamin KO reductase

Table 2. Tissue warfarin distribution and microsomal warfarin content in rats 20 hr following an acute subcutaneous *S*-warfarin dose

Warf. (mg/kg)	Tissue content (ng/g)* Plasma	Liver	Kidney	Testis	Microsomal content (ng/mg)* Liver	Kidney	Testis
0.2 (N = 3)	170 ± 30	230 ± 15	90 ± 15	60 ± 6	9 ± 0.8	—†	—
0.4 (N = 3)	390 ± 30	350 ± 11	110 ± 15	100 ± 10	13 ± 2.5	—	—
1 (N = 3)	1,280 ± 80	870 ± 50	310 ± 60	270 ± 30	13 ± 10	4	6

* Concentrations are given per gram wet tissue weight, or per mg protein content (microsomes). Data are given as mean (±SD). Microsomes of kidney and testis were from pooled organs of three rats.

† No warfarin detectable.

Table 4. The steady state warfarin tissue distribution and the microsomal warfarin content during chronic *S*-warfarin administration*

	Tissue content (ng/g)†				Microsomal content (ng/mg)†		
Exp.	Plasma	Liver	Kidney	Testis	Liver	Kidney	Testis
1	280 ±60	760 ± 100	120 ± 20	140 ± 20	14 ± 2.5	1	4
2	270 ± 60	730 ± 60	140 ± 50	140 ± 10	18 ± 1.9	2.5	6

* *S*-warfarin was administered chronically by means of the Alzet osmotic minipump. Tissue concentrations and liver microsomal content are the means (±SD) of individuals (N = 4). The contents in microsomes of kidney and testis are estimated in microsomes obtained from the pooled organs of 4 rats.

† See Table 2.

activity in kidney and testis microsomes and its sensitivity to warfarin. *In vivo* effects of warfarin on extrahepatic tissues have been shown recently by Roncaglioni *et al.* [25]. Warfarin administered to rats via their drinking water (about 60 μg racemic warfarin per rat per day) induced the accumulation of carboxylatable substrate in liver, lung, spleen and testis [25]. The effect was most pronounced for liver and the least for testis. It is likely to ascribe this accumulation of endogenous substrate to the effect of warfarin on the tissue carboxylases.

Our data unequivocally show that warfarin in "therapeutic" doses affected vitamin KO reductase activity in kidney and testis in the rat. The data also show that the liver system is much more susceptible to warfarin therapy than the other tissues. Most likely this apparent tissue selectivity depends largely on the differences in tissue distribution of warfarin. As was demonstrated during therapeutic steady-state warfarin disposition, the drug showed the highest distribution in the liver. The warfarin content in microsomes run concurrently (Table 4). The reason for the warfarin accumulation in the liver is likely a transporter in the hepatocyte extracting oral anticoagulants from the circulation [26]. Pharmacokinetics determined also the absence of response of kidney and testis in the 0.2 and 0.4 mg/kg single-dose warfarin experiments. Twenty hours following the acute doses, hardly any warfarin had penetrated into kidney or testis (Table 2). However, factors other than mere pharmacokinetics are also involved. This has to be concluded from the observed differences between the acute and chronic effects. Following the 1 mg/kg dose, higher warfarin tissue levels were obtained than following the chronic warfarin administration (Table 2 vs Table 4). Yet, after the acute warfarin administration the effect on vitamin KO reductase was the same (liver and kidney) or even less (testis) than in the chronic experiment (Table 1 vs Table 3). Apparently, the time of exposure is an important factor determining the effect.

As was previously shown by Fasco and Principe [27], the inhibition of liver microsomal vitamin KO reductase activity is a marker for the time course of the warfarin effect on blood clotting activity. Whether the reduction in vitamin KO reductase activity is due to an irreversible inactivation or to a tight almost "irreversible" warfarin binding, is not known [28]. We neither observed vitamin KO reductase activation by extensive washings, nor by overnight dialysis of liver microsomes from warfarin-treated rats (unpublished data). Whereas warfarin was observed in the cytosol (100,000 *g* supernatant) of the liver, no warfarin was detected in any of the 100,000 *g* supernatants of the washings of the microsomes. Yet, as is shown in this study, warfarin was isolated from microsomes following organic solvent extraction. Whether this extractable warfarin reflects the warfarin accountable for the inhibition, however, is not sure.

Should the findings in the rat hold for man too, it is understandable why oral anticoagulants, even when medicated for many years, do not show noticeable effects on tissue functions other than the clotting system. The concentrations of oral anticoagulants in non-hepatic tissues during anticlotting therapy are too low to suppress their vitamin K-dependent systems deep enough to provoke tissue-specific insufficiencies. Evidently the above holds only if vitamin K-dependent proteins are important for non-hepatic tissues to function. To get further insight in the physiological functions of the tissue-specific vitamin K-dependent systems and/or products, vitamin K-antagonists having a more favourable tissue distribution would be of great help. We are currently investigating structure pharmacokinetic relationships of 4-hydroxycoumarins for that purpose.

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