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TISSUE DISTRIBUTION AND WARFARIN SENSITIVITY OF VITAMIN K EPOXIDE REDUCTASE*

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Abstract—The distribution of vitamin K epoxide reductase activity and its sensitivity to warfarin have been examined in whole microsomes from tissues of both control and warfarin-resistant strain rats. The distribution of activity roughly paralleled that previously shown for the vitamin K-dependent carboxylase. Activity on a per gram tissue basis was highest in kidney, adrenal, spleen, lung, testes, and epididymis at a level about 1/20th of that present in liver microsomes. Vitamin K quinone formation by microsomes from warfarin-resistant rats was approximately half that of control strain samples. In addition, hydroxy vitamin K was formed by warfarin-resistant strain microsomes to about the same extent as vitamin K quinone in all tissues. The K_m values for dithiothreitol (DTT) and vitamin K epoxide were similar in all tissues (range = 0.1–0.2 mM DTT at 40 μM vitamin K epoxide, and 10–30 μM vitamin K epoxide at 2 mM DTT). The sensitivities to warfarin were similar for all control strain rat tissues (I_{50} = 10–20 μM at 2 mM DTT and 40 μM vitamin K epoxide) and similarly elevated for all warfarin-resistant rat tissues (I_{50} = 30 to >80 μM). These results suggest that the identical enzyme is expressed in all tissues and that tissue specific ioszymes do not occur.

The occurrence of gamma-carboxyglutamic acid (GLA)‡ was first demonstrated in the vitamin K-dependent coagulation factor, prothrombin, then in Factors VII, IX, and X and in the proteins C, S, and Z. The role of this amino acid in the function of these plasma proteins and the role of vitamin K in the post-translational formation of GLA residues during their hepatic biosynthesis have largely been elucidated [1–3]. A microsomal vitamin K-dependent carboxylase requires vitamin K hydroquinone which is converted to vitamin K epoxide during the oxygen-dependent carboxylation of peptide-bound glutamyl residues in precursors to these proteins. The epoxide is recycled by reduction to the quinone via a warfarin-inhibitable dithiol-dependent microsomal vitamin K epoxide reductase [4] and subsequent reduction to the hydroquinone via both dithiol-dependent [5] and pyridine nucleotide-linked reductases [6]. Inhibition of this cycle by warfarin and other coumarin or indanedione anticoagulants results in accumulation of vitamin K epoxide and cessation of active coagulation factor synthesis [7].

Subsequently, GLA has been demonstrated in total crude protein hydrolyzates, several specific GLA-containing proteins have been identified and their synthesis *in situ* has been demonstrated, and microsomal vitamin K-dependent carboxylation of both endogenous protein substrates and simple peptides has been shown, in a wide variety of tissues [8–12]. Early work on vitamin K epoxidase activity [13], prior to its recognition as a cofunction of the vitamin K-dependent carboxylase, showed a similarly wide distribution. It remains to be determined to what extent vitamin K epoxide generated due to carboxylation in extrahepatic tissues is recycled *in situ* and whether the identical epoxide reductase occurs in all tissues. These results would bear on the extent of inhibition of extrahepatic vitamin K function likely to occur during coumarin anticoagulant treatment.

The occurrence of vitamin K epoxide reductase in extrahepatic tissues may be anticipated from the observation of warfarin-inhibitable microsomal [vitamin K quinone, plus dithiothreitol]-dependent carboxylase activity in previous studies [10, 11]. The required vitamin K hydroquinone is presumably supplied under these conditions via the dithiothreitol-dependent warfarin-inhibitable vitamin K quinone reductase [5] which is very similar, if not identical, to the vitamin K epoxide reductase [14]. This paper examines the tissue distribution and warfarin sensitivity of vitamin K epoxide reductase activity directly.

MATERIALS AND METHODS

Control strain rats were obtained from the Holzman Co. (Madison, WI). Warfarin-resistant strain rats [15] were a gift from Dr. J. W. Suttie (University of Wisconsin-Madison), and a breeding colony was subsequently established. Animals were maintained in wire-bottomed cages and provided stock chow and water *ad lib*. Warfarin-resistant rats were supplied 0.5 mg/l menadione bisulfite in the drinking water. Young adult males (275–325 g) were used in all experiments.

Animals were fasted overnight before use and

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‡ Abbreviations: GLA, gamma-carboxyglutamic acid; Vit K, vitamin K quinone; Vit KHOH, hydroxy vitamin K (2-methyl-3-phytyl-2(or)3-hydroxy-2,3-dihydro-1,4-naphthoquinone); and DTT, dithiothreitol.

killed by decapitation. Tissues from at least six individuals were excised, pooled, rinsed, and weighed, and homogenates were prepared in 2 ml/g tissue of cold 0.25 M sucrose, 10 mM Tris–HCl, 1 mM EDTA buffer, pH 7.6. All tissues, except adrenal, testes, brain, thymus, and liver, were processed with a Tekmar tissumizer, before final processing with a teflon–glass homogenizer. Microsomes were isolated by centrifugation (100,000 $g \times 60$ min) of the supernatant fraction from an initial 10,000 $g \times 10$ min centrifugation step and stored as pellets frozen in liquid nitrogen. Pellets were thawed and resuspended to a concentration equivalent to 0.5 to 2 g tissue/ml in homogenization buffer for assay. Protein concentrations were determined by the method of Lowry *et al.* [16] in the presence of 0.5% sodium cholate to solubilize membrane-bound material, and bovine serum albumin was used as the standard.

Vitamin K epoxide reductase activity was assayed using 0.5 ml of resuspended microsomes, warfarin added in 10 μl of ethanol, and vitamin K epoxide added in 5 μl of 2% Tween-80, assembled in order on ice. Following a 2-min preincubation at 23°, reactions were initiated by addition of dithiothreitol in 20 μl of buffer. Concentrations equivalent to 2 g tissue/ml for pancreas, brain, heart, thymus, muscle, stomach, and intestine, 0.5 g tissue/ml for liver, and 1 g tissue/ml for other tissues, 40 μM vitamin K epoxide, and 2 mM dithiothreitol, and 30-min incubations (10 min for liver) were used unless otherwise stated.

The reactions were terminated by addition of 0.05 ml of 2 M sodium phosphate buffer, pH 6, and 2 vol. of isopropanol/hexane (1:1). The hexane phase was evaporated and redissolved in 100 μl of methanol; 50 μl was injected for analysis by reverse phase HPLC. Vitamin K metabolites were separated on an Alltech 10 μm C18 column (250 × 4 mm) at a flow rate of 2 ml/min of 15% isopropanol/85% methanol and quantitated by UV absorption at 254 nm as previously described [17]. Retention times of 3.03 min (hydroxy vitamin K), 4.38 min (vitamin K epoxide), and 6.20 min (vitamin K quinone) were observed. Using these methods, any vitamin K hydroquinone formed during the incubations is recovered as vitamin K quinone. Run times up to 15 min were required in some cases to avoid overlap of late eluting endogenous materials.

Values for the K_m of vitamin K epoxide were determined from incubations at 5, 10, 20, 40, and 60 μM vitamin and 2 mM DTT. Values for the K_m of DTT were determined from incubations at 0.05, 0.1, 0.2, 0.5, 1, and 2 mM DTT and 40 μM vitamin K epoxide. The data were fit to the hyperbolic form of the Michaelis–Menten equation [18]. Values for the concentration of warfarin producing 50% inhibition (I_{50}) were determined from incubations at 2, 5, 10, 20, 40, and 80 μM warfarin, 40 μM vitamin K epoxide, and 2 mM DTT. Incubations for control and warfarin-resistant rat tissue samples were performed in parallel, and values of I_{50} were extrapolated from plots of percent control activity versus log warfarin concentration.

Vitamin K (Sigma Chemical Co., St. Louis, MO) was converted to vitamin K epoxide, and both materials were purified by preparative HPLC as previously described [17]. Dithiothreitol was from Calbiochem, San Diego, CA. Warfarin (Lot No. 90691) and menadione bisulfite were from ICN Pharmaceuticals, Plainview, NY.

RESULTS

Microsomal vitamin K epoxide reductase activity could be detected in most tissues under the same conditions used for assay of the activity in rat liver microsomes. The HPLC method employed provided adequate resolution of vitamin K metabolites from endogenous materials in all tissues except epididymis. (The hydroxy vitamin K peak was obscured and small contributions to the area for vitamin K epoxide were observed in this case.) No endogenous materials ran at the same retention time as vitamin K quinone in any of the samples tested. The HPLC limit of detection was 0.01 nmol of vitamin K quinone injected, corresponding to a minimum detectable activity of 0.67 pmol/min/g tissue at the highest concentration (2 g tissue/ml) and longest incubation times tested (30 min). Conditions for optimal activity of microsomes from each tissue were similar to those for liver, and a single standard set of conditions (40 μM vitamin K epoxide, 2 mM DTT, 23°, and pH 7.6) was chosen as the basis for comparison. Preliminary studies with selected tissues (liver, testes, and brain) showed that the amount of vitamin K formed increased linearly with incubation time up to 30 min and enzyme concentration in the range 0.65 to 23 mg microsomal protein/ml.

Appreciable activity was detectable in adrenal, kidney, spleen, testes, epididymis, and lung at a level approximately 1/20th of that found in liver on a per gram basis (Table 1). A lower, but readily detectable level of activity (>4 × detection limit) was found in brain, thymus, prostate, skeletal muscle, heart, and epididymal fat pads. Activity was detectable in pancreas (1 nmol/g tissue/30 min) only if the microsomes were prepared in the presence of 0.1 mM tosyl-lysine chloromethyl ketone (TLCK) and 0.1 mM phenylmethanesulfonyl fluoride (PMSF). The addition of protease inhibitors did not affect the level of activity detected in other tissues. No activity was detectable in whole stomach and small intestine segments even when protease inhibitors were included during preparation of the microsomes. The activities per mg microsomal protein were similar (range = 3.5–7 pmol/min/mg protein) for most tissues other than liver (39 pmol/min/mg protein). Differences in the activity per g tissue reflected primarily variations in yield of microsomal protein. Exceptions included adrenals, epididymis, and epididymal fat pads (sp. act. = 13, 24, and 44 pmol/min/mg protein respectively).

Vitamin K quinone formation by microsomes from warfarin-resistant strain rats was approximately one-half that observed in microsomes from control strain rats in all tissues. The yield of total microsomal protein (mg/g tissue) was the same in both strains, indicating that the observed differences in activity were specific. In addition to vitamin K quinone, hydroxy vitamin K was observed as a product of

Table 1. Distribution of vitamin K epoxide reductase activity in control and warfarin-resistant strain rats*

Tissue	mg protein† g tissue	Vit K epoxide reductase activity (nmol/g tissue/30 min) Control‡ Vit K	Warf-resistant§ Vit K	Warf-resistant§ Vit KHOH
Liver	23.5	27.6	13.1	10.4
Adrenal	4.3	1.7	0.80	1.4
Kidney	16.1	1.7	0.82	0.89
Testes	6.0	1.3	0.76	0.94
Epididymis	3.0	2.2	1.14	–
Prostate	1.4	0.24	0.17	+
Spleen	10.3	1.1	0.31	0.36
Lung	8.6	1.2	0.53	0.84
Brain	2.6	0.44	0.21	0.28
Thymus	3.6	0.12	0.09	+
Heart	1.3	0.21	0.12	+
Muscle	2.0	0.11	0.14	+
Fat pads	1.2	0.57	0.11	0.15
Pancreas	7.0	1.0	–	–
Stomach	2.0	0.0	–	–
Intestine	3.3	0.0	–	–

* Activity was assayed in whole microsomes at 40 μM vitamin K epoxide and 2 mM DTT (average for replicate preparations differing by at most 20%).

† Milligrams microsomal protein per gram tissue ± 0.5 mg/g. Values for microsomes from control and warfarin-resistant strain rats were identical within experimental error.

‡ Nanomoles vitamin K quinone produced by microsomes from control strain rats.

§ Nanomoles vitamin K quinone produced (Vit K) and nmol hydroxy vitamin K (Vit KHOH) produced by warfarin resistant rat strain microsomes, only. (+) Present, but close to limit of detection and could not be accurately quantitated.

vitamin K epoxide metabolism by microsomes from all warfarin-resistant rat tissues at a level comparable to vitamin K quinone. No hydroxy vitamin K was produced by microsomes from control strain rat tissues.

Table 2. Substrate K_m values of vitamin K epoxide reductase*

Tissue	K_m (μM) Vit KO	K_m (mM) DTT
Liver	16 ± 1	0.10 ± 0.01
Kidney	35 ± 4	0.18 ± 0.03
Testes	23 ± 3	0.08 ± 0.01
Epididymis	13 ± 2	0.21 ± 0.02
Prostate	25 ± 5	0.20 ± 0.04
Spleen	12 ± 1	0.15 ± 0.04
Lung	28 ± 5	0.12 ± 0.01
Brain	21 ± 3	0.08 ± 0.01
Thymus	17 ± 1	0.09 ± 0.04
Heart	13 ± 2	0.14 ± 0.03
Muscle	32 ± 3	0.21 ± 0.17
Fat pads	23 ± 4	0.09 ± 0.01

* Apparent K_m values for vitamin K epoxide (Vit KO) at 2 mM DTT and for dithiothreitol (DTT) at 40 μM vitamin K epoxide in whole microsomes. Average of best fit values ± combined standard estimate of error, plus preparation to preparation variation.

The K_m values for dithiothreitol and vitamin K epoxide were determined using microsomes from control strain rats (Table 2). Double-reciprocal plots of 1/v versus 1/[KO] at 2 mM DTT were linear in the range from 2 to 40 μM vitamin K epoxide. A lower than expected rate was observed at 60 μM vitamin K epoxide which approaches the limit of solubility of the vitamin in the Tween-80 stock solution used to add the substrate. Fit values of the K_m for vitamin K epoxide were similar in all tissues (range = 12–32 μM). Double-reciprocal plots of 1/v versus 1/[DTT] were linear over the range 0.05–2 mM DTT. Some substrate inhibition was observed at 4 mM DTT. Fit values of K_m for DTT were also similar for all tissues (range = 0.1–0.2 mM DTT). An obvious major variation in assays for microsomes from different tissues is the variation in total protein (and lipid) concentration for assays conducted within the limited practical range of tissue concentrations (0.5 to 2 g tissue/ml). Tests using liver microsomes showed that the effect of total protein concentration on the observed values of K_m for DTT and vitamin K epoxide was negligible in the relevant total protein range. The observed variation among tissues is probably real; however, the magnitudes of the differences do not suggest any major change in the enzyme.

The sensitivity of vitamin K epoxide reductase to warfarin inhibition was determined using microsomes from both control and warfarin-resistant strain rats (Table 3). The mechanism of warfarin inhibition

Table 3. Warfarin sensitivity of vitamin K epoxide reductase*

	I_{50} Warf† (μM)		% Inhibition at 20 μM Warf‡		
			Vit K		Vit KHOH
Tissue	Control	WR	Control	WR	WR only
Liver	10	28	74	43	78
Adrenal	–	–	53	29	47
Kidney	15	80	59	14	20
Testes	17	80	55	15	23
Epididymis	24	80	35	0	–
Prostate	7	–	80	43	–
Spleen	9	80	80	9	20
Lung	15	69	59	36	18
Brain	11	50	64	37	17
Thymus	–	–	73	40	–
Heart	11	68	77	23	–
Muscle	8	40	75	41	–
Fat pads	12	60	66	36	20

* Warfarin inhibition of vitamin K epoxide reductase activity in whole microsomes from control or warfarin-resistant (WR) strain rats at 40 μM vitamin K epoxide and 2 mM DTT.

† Concentration of warfarin producing 50% inhibition of vitamin K quinone formation.

‡ Percent inhibition of vitamin K quinone formation (Vit K) or of hydroxy vitamin K formation (Vit KHOH, in warfarin-resistant strain microsomes, only) at a single fixed warfarin concentration (20 μM).

is not known in detail. Using whole microsomes, the extent of inhibition does not fit a simple straight line on Dixon plots (1/v vs [warfarin]). Sensitivity to the inhibitor is a function of both substrate concentrations, but the steady-state kinetic patterns cannot be established using intact microsomal preparations; therefore, no attempt was made to determine K_i values. For comparative purposes, the same standard incubation conditions used for determination of total activity were chosen for these experiments as well. Under these conditions, the difference between the sensitivities of the activities in liver microsomes from the two strains was readily detected. The sensitivity to warfarin, expressed either as the percent inhibition at a single fixed warfarin concentration (20 μM) or the concentration of warfarin producing 50% inhibition (I_{50}) varied somewhat for various tissues (range = 8–24 μM for microsomes from control strain rats) with most tissues clustered about 10 μM. Again, a possible source of variation is the varying concentration of protein used for various tissues. Tests using control rat liver microsomes again showed a negligible variation of the I_{50} value with protein concentration. The sensitivity of the enzyme in epididymis, testes, and possibly kidney and lung may vary significantly from that of liver and the other tissues; however, the magnitudes of these differences do not suggest major differences in the enzyme.

The sensitivity of the enzyme in microsomes from warfarin-resistant strain rats was uniformly lower than for microsomes from the corresponding tissues of control strain rats. The tissue to tissue variation was more significant than for control rat microsomes when expressed as absolute numbers, but the fold increase in I_{50} values was similar. All differences between the control and warfarin-resistant strain values were meaningful. Hydroxy vitamin K formation by warfarin-resistant strain microsomes was also inhibited by warfarin.

DISCUSSION

The results show that vitamin K epoxide reductase activity is widely distributed among almost all tissues. Several studies have appeared while this work was in progress supporting this conclusion. Epoxide reductase was demonstrated in kidney and testes microsomes (1.0 and 1.8 pmol K/min/mg protein compared to 13 pmol/min/mg for liver microsomes) in one study [19], and in kidney, testes, lung, spleen, and brain microsomes at levels of 15, 16, 7, 5, and 6 pmol/min/mg compared to 28 pmol/min/mg for liver microsomes in a second study from the same group [20]. With the exception of the significantly higher level of activity in kidney and testes microsomes in the second study, these results are in reasonable agreement with the work presented here (kidney, 3.5; testes, 7; lung, 4.5; spleen, 3.5; brain, 5.3; and liver, 39 pmol/min/mg protein). The variation in the actual levels reported may reflect differences in the methods for preparation of microsomes and may be more marked for activity expressed on a per mg protein basis. A comparison of activities on a per gram tissue basis cannot be made from the data presented in the other studies. There are numerous differences between all three studies in the detailed methods for the incubation and analyses, the significance of which are not yet ascertainable.

Epoxide reductase activity has also been demonstrated in several cancer cell culture lines of nonhepatic origin [21, 22]. A direct comparison with our data cannot be made from the data reported.

The distribution of vitamin K epoxide reductase

activity reported here and in the above studies [19, 20] roughly parallels that previously reported by other workers for microsomal vitamin K-dependent carboxylation [10–12] and the related vitamin K epoxidase activity [13]. This suggests that regeneration of epoxide can occur *in situ* without required circulation of the vitamin for regeneration in liver. Although when both activity and organ size are taken into account liver is responsible for greater than 95% of the total activity of the internal organs, it is probable that regeneration of the vitamin in each individual organ is essential for continued carboxylation in that organ. Vitamin K metabolism related to vitamin K-dependent carboxylation in liver is a highly localized phenomenon occurring in the rough endoplasmic reticulum [23], and the metabolically active pool probably involves a fraction of the total vitamin. The same situation is likely to apply in all tissues in which gamma-carboxyglutamate residues are formed. The high specific activity of vitamin K epoxide reductase in liver is consistent with an endoplasmic reticulum differentiated to support the high rate of vitamin K metabolism necessary for its role in the large scale synthesis of blood coagulation factors. In other tissues, the specific activity is much lower, in accord with their much lower scale of vitamin K-dependent protein synthesis.

A substantial and variable fraction of the total body activity of vitamin K epoxide reductase may also be associated with adipose tissue. Vitamin K-dependent carboxylation has not been observed in adipose tissue. The high specific activity of vitamin K epoxide reductase in adipose microsomes may suggest an unusual role for this enzyme in vitamin K storage, rather than vitamin K-dependent protein synthesis.

Vitamin K epoxide reductase in liver has been shown to be the target of the coumarin and indanedione anticoagulants [7, 24]. To the extent that extrahepatic vitamin K epoxide reductase resembles the liver enzyme, some degree of inhibition of extrahepatic vitamin K-dependent protein synthesis may be expected during anticoagulant treatment. The present results show that the activity in microsomes from all tissues is sensitive to warfarin *in vitro*. Other studies show that the activity in various tissues is also sensitive to warfarin administration *in vivo* [19–22].

Warfarin resistance has been shown to be inherited as a single autosomal mutation [15, 25]. Classification of the individuals in these genetic studies has been based on changes in coagulation factor levels in response to a warfarin challenge and reflects effects on hepatic vitamin K function. The basis for warfarin resistance has been shown to be a decrease in the sensitivity of the hepatic vitamin K epoxide reductase to warfarin [24]. The results show that the enzyme in all other tissues is also less sensitive to warfarin in the mutant strain than in the control strain.

A characteristic of the warfarin-resistant strain is an elevated requirement for vitamin K [15] which may relate to the consistently observed lower activity of vitamin K quinone formation in hepatic microsomes from resistant versus control strain rats [22, 26–28]. The results show a lower level of vitamin K quinone formation by microsomes from all tissues of warfarin resistant rats. A second characteristic of the mutant enzyme is the formation of hydroxy vitamin K as a product of an abnormal reaction mechanism [17, 29]. The results show that this metabolite is also formed by microsomes from all tissues of warfarin-resistant rats, but not by microsomes from control strain rats.

These results suggest that the identical enzyme is expressed in all tissues from the same wild type or mutant gene. This conclusion is supported by the similarity of the K_m values for the substrates and the I_{50} values for the inhibitor warfarin for the enzyme in microsomes from all tissues. A consequence is the virtual certainty of side effects of anticoagulants acting through inhibition of hepatic vitamin K epoxide reductase due to inhibition of the enzyme in other tissues *in vivo*, though such effects may be unobservably small or slow in developing. The identity of the enzyme in all tissues implies that attempts to develop tissue specific inhibitors based on differences in tissue specific isozyme mechanisms cannot be undertaken. Attempts to improve the tissue specificity of vitamin K antagonists must rely solely on differences in uptake and metabolism.

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