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TISSUE DISTRIBUTION OF SELECTIVE WARFARIN BINDING SITES IN THE RAT

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Abstract—Liver microsomes contain a specific warfarin binding site that is related to the target enzyme vitamin KO reductase [Thijssen HHW and Baars LGM, *Biochem Pharmacol* **38**: 1115–1120, 1989]. In this study the distribution of the warfarin binder in the rat was investigated. Rats were given tracer doses of [^{14}C]warfarin and tissue distribution was estimated after a time period. The selectivity of the distribution was verified by the ability of unlabeled warfarin to displace *in vivo* the tissue accumulated [^{14}C]warfarin. The relation to the target enzyme vitamin KO reductase was verified by comparing the results with distribution behavior in the Scottish warfarin-resistant rat strain. The results show that in addition to liver various non-hepatic tissues accumulate warfarin. Among the tissues having a high accumulation ratio and a high rate of exchange by unlabeled warfarin are liver, pancreas, kidney, and salivary gland. Also arteria (aorta), bone, lung and spleen show exchangable [^{14}C]warfarin accumulation. In HS rats the [^{14}C]warfarin distribution was affected similarly for all tissues; lower levels of accumulation and higher rates of exchange by unlabeled warfarin. The tissue-bound warfarin was recovered predominantly in the microsomal fraction. Its release could only be accomplished in the presence of dithiothreitol and appeared to be stereoselective. The *in vivo* distribution pattern correlated with the number of warfarin binding sites in the tissue microsomes. The microsomal vitamin KO reductase activity did not always correlate to the binding capacity. The distribution was not affected by vitamin K deficiency. Warfarin-treated rats showed vitamin K epoxide accumulation in most of the organs having the warfarin binder.

It is well established that rat liver tissue contains a binding site for 4-hydroxycoumarin anticoagulants [1–3]. This binding site, located in the microsomal fraction, determines the initial rapid distribution phase of i.v. administered warfarin and the extended period of elimination [4–7]. More recently we showed that the microsomal warfarin binding is related to the target enzyme vitamin K epoxide reductase [3]. The enzyme is a dithiol dependent reductase which regains vitamin K from its 2,3-epoxide metabolite (vitamin KO) and probably also reduces vitamin K to the hydroquinone form. The hydroquinone functions as a cofactor in a carboxylation reaction which transforms glutamic acid residues of clotting factor precursor proteins to γ-carboxy glutamic acids (Gla†). Concomitantly, the hydroquinone is transformed to the epoxide. Epoxidation and reduction are apparently closely linked to ensure an efficient recycling of vitamin K. Inhibition of the reductase(s) by the 4-hydroxycoumarins leads to the exhaustion of the hydroquinone cofactor, hence the synthesis of the particular clotting factors is reduced (for reviews, see Refs 8 and 9). The precise mechanism of the interaction of the 4-hydroxycoumarins with the target enzyme is still poorly understood. However, there is substantial evidence that the coumarins interact with the inactive (=oxidized) form of the enzyme in an apparently irreversible way [10, 11]. Activation of the enzyme by the as yet unknown endogenous reductant (*ex-vivo* by DTT) reverses the binding. The activation step, however, is strongly retarded by the bound inhibitor [6, 11, 12].

Elements of the vitamin K cycle (e.g. the vitamin K-dependent carboxylase and vitamin KO reductase) are found to be present in extra-hepatic tissues such as lung, bone, kidney, spleen and testis [13–16]. Any functions of these non-hepatic vitamin K-dependent activities are still unknown. However, it has been suggested that the matrix Gla protein may be a common product [17]. Both, matrix Gla protein and bone Gla protein (= osteocalcin) are vitamin K-dependent proteins present abundantly in bone [18, 19].

The presence of the vitamin K cycle is established mainly from *in vitro* enzyme assays. However, enzyme activities in crude tissue preparations may not correspond to the true tissue distribution if the optimal assay conditions vary with tissue; for instance, rat testis vitamin KO reductase activity has been reported to be 5 [20], 10 [16], and 57% [21] of liver vitamin KO reductase.

We investigated the selective distribution in the rat of tracer doses of [^{14}C]warfarin. The ability to displace warfarin from its tissue binding site with unlabeled warfarin is an indicator that the vitamin K cycle is operating. Tissue distribution is compared with vitamin KO reductase activity and microsomal binding capacity. The results show that exocrine organs in particular selectively accumulate warfarin.

MATERIALS AND METHODS

Male Wistar rats and male warfarin resistant HS

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† Abbreviations: Gla, γ-carboxy glutamic acid; DTT, dithiothreitol.

rats, both 220–270 g, were used. The HS rats [11] are bred in the laboratory animal facility center of our faculty. The animals had free access to food and tap water and were housed under a 12 hr light/dark cycle. To obtain vitamin K-deficient animals they were fed on a vitamin K deficient diet (Hope Farm, Woerden, The Netherlands). Rats (two groups of 3–4 animals) were given tracer doses of *rac* [^{14}C]warfarin (1.5 μCi = 32 nmol; Amersham, The Netherlands) subcutaneously (s.c.). After a period of time (> 40 hr, see results) *S*-warfarin, 5 mg/kg s.c., was administered; control rats were given saline. Following another time period rats were anesthetized with ether, the abdomen was opened and blood was withdrawn as completely as possible via the abdominal aorta. The body was perfused with 40 mL of saline via the left ventricle whereafter tissues were excised. Tissues were homogenized in 3 vol. of 0.02 M Tris–HCl buffer, pH 7.4, containing 0.15 M KCl and 0.25 M sucrose. Arterial tissue (aorta, carotis) and bone tissue were solubilized in soluene-350 (Packard, Amstelveen, The Netherlands). ^{14}C-Radioactivity was estimated by liquid scintillation counting.

Tissue microsomes were prepared by fractional centrifugation. For the preparation of microsomes of pancreas and salivary (submaxillary) gland tissue the homogenizing buffer contained 0.1 mg aprotinin and 0.2 mg soybean chymotrypsin inhibitor (Boehringer, F.R.G.). Liver tissue mitochondria were isolated according to established procedures [22].

Microsomal vitamin KO reductase activity and microsomal warfarin binding were assayed as described previously [3].

The enantiomeric composition of [^{14}C]warfarin in liver microsomes was estimated following the separation of the diastereomeric Boc-L-proline esters of warfarin [23]. Briefly, after its extraction [^{14}C]-warfarin, together with unlabeled *rac*-warfarin, was esterified with Boc-L-proline in the presence of dicyclohexyldicarbodiimide. Separation of the diasteriomers was performed by silica gel thin layer chromatography (silica gel plates with concentration zone, Merck, Darmstadt, F.R.G.) using petroleum ether, ethylacetate, methanol (75:25:1) as developing solvent.

Tissue distribution of vitamin K and vitamin KO. Rats received saline (1 mL/kg, controls, N = 3) or *S*-warfarin (5 mg/kg in saline, N = 3) subcutaneously. Vitamin K (10 mg/kg, Konakion, Roche, Basel, Switzerland) was administered i.v. 16 hr later. The rats were killed 1.5 hr after vitamin K administration. Under light ether anesthesia the rats were bled from the abdominal aorta and the body was perfused with 40 mL saline via the left ventricle. Tissues were excised and homogenized in saline (1:3). Tissue vitamin K and vitamin KO were assayed by HPLC following organic solvent extraction. Briefly, 0.2 mL of tissue homogenate was mixed with 2.8 mL of a water–iso-propanol mixture (2:3, v/v) containing 5 μg of tocopherol acetate as internal standard. The mixture was extracted twice with 1.5 mL of *n*-hexane. The hexane phase was evaporated to dryness and the residue was taken up in 50 μL of iso-propanol. Vitamin KO, internal standard, and vitamin K were separated and quantified on a Lichrosphere RP18 column (10 × 3 mm; Chrompack, The Netherlands) using a mixture of acetonitrile, iso-propanol and water (100:8:1) as eluting solvent. UV monitoring was at 250 nm.

RESULTS

Tissue distribution in Wistar rats

The tissue distribution of label following microdoses of [^{14}C]warfarin to male Wistar rats is presented in Table 1. In relation to the circulation (plasma), accumulation of label was seen in various tissues. For some of the tissues, i.e. liver, kidney, lung, pancreas, testis and plasma, the identiy of the radioactivity was checked by high performance liquid chromatography after organic solvent extraction; more than 80–90% of the tissue radioactivity was recovered under the warfarin peak. Liver showed the highest accumulation ratio, the liver to plasma ratio being greater than 40. Moreover, 70–72 hr after warfarin administration the amount recovered in liver tissue was about 30–40% of the dose. Other organs with high warfarin accumulation ratios included pancreas (60–70% of liver ratio), kidney (30% of liver ratio), submaxillary and parotid (not shown) glands (19% of liver ratio) and carotis (not shown). Muscle tissue showed no accumulation. Distribution in brain tissue was weak, the brain to plasma ratio being 0.5. Also, fat tissue (data not shown) showed hardly any radioactivity.

There was no essential difference in warfarin distribution between rats fed normal lab food and rats fed on a vitamin K-deficient diet for more than 10 days (thrombo-test values in these rats were between 15 and 25% of normal).

The subcellular distribution of tissue radioactivity was estimated for liver, kidney, lung, pancreas, submaxillary gland and testis (Table 2). Assuming that the 800 *g* pellet (liver) or 10,000 *g* pellet (other tissues) contained cell debris including significant amounts of entrapped ER fragments [24], the data show that the microsomal fraction contained most of the tissue bound [^{14}C]warfarin. Cytosol and mitochondria each contained 5% or less of the tissue radioactivity. For the kidney, the distribution in the cortex was twice as high as in the medulla.

To estimate the type of warfarin binding, liver microsomes obtained in the experiments were subjected to dialysis against 10 vol. of buffer containing unlabeled warfarin with and without 50 mM DTT (Fig 1). In the absence of DTT 6 hr of dialysis at room temperature released less than 10% of the radioactivity. In the presence of DTT about 30% was released. Furthermore, [^{14}C]warfarin in the isolated microsomes appeared to be racemic. After dialysis against DTT the *R*/*S* ratio in the microsomes was changed to about 0.5, indicating that it was mostly the *R* enantiomer that was released. Such dialysis experiments were performed also with microsomes of kidney and pancreas; both tissues only showed appreciable [^{14}C]warfarin release in the presence of DTT.

In vivo *displacement*

The administration of *S*-warfarin (5 mg/kg, s.c.) displaced accumulated warfarin in most of the tissues

Table 1. [^{14}C]Warfarin tissue distribution in control rats and after the administration of unlabeled warfarin

Tissue	64 + 6 (hr) Control [dpm/g tissue (×1000)]	64 + 6 (hr) Displaced [dpm/g tissue (×1000)]	64 + 6 (hr) *d/c*	56 + 16 (hr) Control [dpm/g tissue (×1000)]	56 + 16 (hr) Displaced [dpm/g tissue (×1000)]	56 + 16 (hr) *d/c*
Liver	92.9 ± 0.7	44.9 ± 1.7	0.48	92.4 ± 3.2	26.1 ± 3.1	0.28
Pancreas	61.7 ± 1.1	23.7 ± 1.4	0.38	62.5 ± 2.5	7.6 ± 0.2	0.12
Kidney	27.4 ± 1.9	10.8 ± 0.8	0.39	26.9 ± 0.9	6.9 ± 0.5	0.26
Submaxillary	17.5 ± 0.3	6.9 ± 0.5	0.39	17.7 ± 1.0	2.6 ± 0.1	0.15
Aorta	11.5 ± 0.4	8.4 ± 0.2	0.73	12.4 ± 0.9	6.2 ± 0.1	0.50
Testis	9.6 ± 0.4	8.0 ± 0.5	0.84	9.2 ± 0.3	8.5 ± 0.3	0.93
Lung	8.0 ± 0.8	6.2 ± 0.4	0.77	8.4 ± 1.0	4.0 ± 0.2	0.48
Tibia	6.7 ± 0.4	4.3 ± 0.3	0.65	7.3 ± 0.1	2.3 ± 0.1	0.32
Sternum	6.2 ± 0.2	3.5 ± 0.3	0.57	6.4 ± 0.2	2.1 ± 0.0	0.32
Intestine	7.0 ± 0.9	4.8 ± 0.7	0.69	6.6 ± 0.6	4.7 ± 0.7	0.71
Skin	7.2 ± 0.7	7.0 ± 0.6	0.97	6.7 ± 0.4	6.2 ± 0.7	0.94
Spleen	7.2 ± 0.8	5.5 ± 0.7	0.76	6.5 ± 0.6	3.8 ± 0.4	0.59
Muscle	2.8 ± 0.2	2.2 ± 0.1	0.77	2.0 ± 0.0	1.7 ± 0.2	0.86
Brain	1.0 ± 0.0	0.8 ± 0.0	0.86	0.9 ± 0.0	0.7 ± 0.0	0.81
Heart	3.2 ± 0.2	4.6 ± 0.6	1.44	2.2 ± 0.2	4.1 ± 0.4	1.85
Plasma	2.3 ± 0.0	17.2 ± 1.0	7.48	2.2 ± 0.1	19.2 ± 0.8	8.72

Rats (N = 3–4) were given 1.5 μCi [^{14}C]warfarin s.c. at time t = 0 hr. At t = 56 and 64 hr, rats were given saline (controls) or 5 mg/kg *S*-warfarin (displaced) s.c. Tissue distribution was estimated 16 and 6 hr after t = 56 and 64 hr, respectively. Data are the means ± SD, *d/c*, ratio displaced over control.

Table 2. The subcellular distribution of [^{14}C]warfarin in rat tissue

Cellular fraction	Liver	Kidney	Lung	Pancreas	Sub-maxillary	Testis
800 *g* pellet	48	ND	ND	ND	ND	ND
10,000 *g* pellet	6*	53	40	28	37	38
Cytosol	2.5	7	2	3.5	8	3
Microsomes	41	42	52	68	54	55

Data are expressed as % of total tissue radioactivity. Tissues were pooled from two rats.
* The mitochondrial fraction of the liver was obtained from the first 10,000 *g* pellet after repeated washing and fractional centrifugation as described previously [22].
ND, not done.

and caused the plasma radioactivity to rise 7–9-fold (Table 1). However, there were clear differences between the various tissues in the rate the accumulated [^{14}C]warfarin was displaced; pancreas, submaxillary gland, and kidney showed the highest rate of release (about 60% was displaced within 6 hr). No release was seen for skin and release was marginal for testis, muscle and brain. Heart tissue showed increased radioactivity but this was probably due to residual blood. Sixteen hours after the administration of unlabeled warfarin, more than 80% of the label in pancreas and submaxillary gland tissue was displaced. This probably holds for the kidney also, as at that time about 30% of the residual radioactivity was not extractable. About 70% of the liver and bone [^{14}C]warfarin stores had been exchanged after 16 hr. Other organs showed a slower rate of displacement (Table 1).

Distribution and displacement in warfarin resistant HS rats

The target enzyme vitamin KO reductase of the warfarin resistant HS rat (the Scottish genotype of resistance) is altered in such a way that its complex with warfarin is still accessible for activation by the (endogenous) reductant [11]. Activation leads to the release of the bound inhibitor. Data for warfarin tissue distribution and the effect of displacement in the HS rat are given in Table 3. In contrast to the sensitive rat, plasma levels are high and tissue levels low. Forty-four hours after the administration of the warfarin tracer only liver tissue showed levels reasonably higher than those in plasma, the liver to plasma ratio being about 2. The distribution in the non-hepatic tissues was about equal (kidney, pancreas) to plasma or lower. Tissue [^{14}C]warfarin

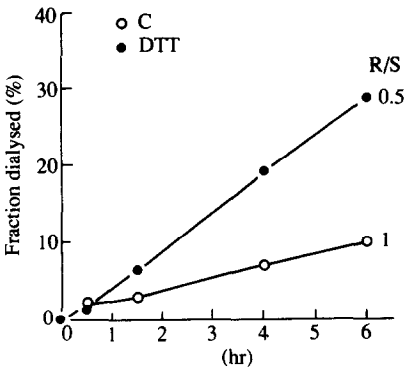

Fig. 1. The release, by dialysis, of rat liver microsomal bound [^{14}C]warfarin. Liver microsomes were prepared from rats given 1.5 μCi [^{14}C]warfarin 72 hr before. Microsomes (20 mg protein/mL) were dialysed against 10 vol. of phosphate buffered saline containing 20 μg/mL unlabeled *rac*-warfarin with (●) and without (○) 50 mM DTT. *R/S* is the enantiomeric ratio (see text). The data are means of an experiment in duplicate.

Table 3. [^{14}C]Warfarin distribution in warfarin-resistant HS rats

Tissue	Control [dpm/g tissue (×1000)]	Displaced	*d/c*
Liver	13.3 ± 1.2	5.2 ± 0.0	0.40
Kidney	7.9 ± 0.7	3.4 ± 0.8	0.43
Pancreas	4.4 ± 0.3	0.7 ± 0.1	0.16
Submaxillary	3.4 ± 0.4	0.7 ± 0.2	0.20
Testis	3.0 ± 0.4	1.7 ± 0.3	0.56
Aorta	0.8 ± 0.2	0.4 ± 0.1	0.46
Sternum	0.8 ± 0.1	0.3 ± 0.0	0.38
Spleen	0.5 ± 0.0	0.7 ± 0.1	1.25
Plasma	6.1 ± 0.2	8.9 ± 0.2	1.46

Rats (N = 3) were given 1.5 μCi [^{14}C]warfarin. At 40 hr, rats were given saline (controls) or 5 mg/kg *S*-warfarin (displaced) s.c. Tissue distribution was estimated 4 hr later. Data are the means ± SD.

could be displaced by unlabeled warfarin. The rate of displacement was much higher in the HS rat; 60–80% within 4 hr for the sensitive tissue. Again, pancreas and submaxillary gland tissue showed the highest rate of displacement.

Tissue microsomal warfarin binding and vitamin K cycle

Microsomal warfarin binding and microsomal vitamin KO reductase activity of some tissues of the Wistar rat are given in Table 4. As is seen, liver microsomes showed the highest warfarin binding capacity, about 60 pmol/mg protein, and the highest reductase activity, about 735 pmol/mg protein/min. The number of binding sites estimated for the non-hepatic tissues agrees reasonably well with the *in vivo* distribution (Table 1). The observed microsomal vitamin KO reductase activity in the tissues was found to be less than 10% of the liver activity.

Table 4. Vitamin KO reductase and warfarin binding capacity in tissue microsomes of Wistar rats

Tissue	Vitamin KO reductase	Warfarin binding
Liver	100	100
Kidney	9.5	29
Testis	5.5	14.7
Lung	8.3	21
Pancreas	9.4	73
Submaxillary	6.6	9.7

Tissues of two rats were pooled.

Data are the means of experiments in duplicate. They are expressed relative to liver (= 100%). Liver vitamin KO reductase activity was 735 pmol vitamin K/mg/min; liver warfarin binding capacity was 60 pmol/mg protein.

Tissue vitamin K and vitamin KO distribution

The vitamin K and vitamin KO distribution in controls and warfarin-treated rats is summarized in Table 5. Following a pharmacological vitamin K dose, distribution at 90 min was mainly to the liver, a 10-fold accumulation over plasma being found. Tissues like pancreas, submaxillaris, and testis showed levels of 10% or less of the plasma levels. Vitamin KO was found to be present in plasma, liver, and lung (4–20% of the vitamin K levels). Warfarin pretreatment resulted in lower vitamin K but higher vitamin KO levels. This was true particularly for liver and lung. For pancreas and testis only one out of the three samples showed detectable vitamin KO levels. No vitamin KO was detected in the submaxillary gland. The fact that total vitamin K + vitamin KO in warfarin-treated rats was lower than in controls, as has also been observed by others [25], was due to the blockade of the recycling of vitamin K and vitamin KO resulting in more being eliminated by other routes [26].

DISCUSSION

The study gives evidence that, in addition to the liver, warfarin binding site(s) are present in various non-hepatic tissues. Along several experimental lines it is established that liver warfarin binding is selective for 4-hydroxycoumarins and is saturable [3, 6]. Saturable warfarin binding in the rat kidney was concluded recently from pharmacokinetic analysis of the warfarin distribution with time [7]. Although saturability and selectivity of the warfarin binding in the other tissues was not tested for explicitly, support for these characteristics is given by the following: (1) accumulated [^{14}C]warfarin can be displaced by unlabeled warfarin and (2) the [^{14}C]-warfarin tissue distribution in the warfarin resistant HS rat is typically affected, showing the tissue distribution of warfarin to be genetically determined.

The liver warfarin binding site is located in the microsomal fraction (present study see Refs 1 and 2) and, as was shown recently, is associated with the target enzyme vitamin KO reductase [3]. Several non-hepatic tissues have been shown to possess enzymes of the vitamin K cycle and thus tissue

Table 5. Tissue distribution of vitamin K and vitamin KO; the effect of warfarin

Tissue	Control (N = 3)		Warfarin-treated (N = 3)	
	Vitamin KO	Vitamin K	Vitamin KO	Vitamin K
Liver	4.2 ± 1.1	108.4 ± 7.3	11.6 ± 5.4	34.4 ± 8.5
Kidney	0.3 ± 0.6*	2.6 ± 1.1	0.3 ± 0.4†	2.7 ± 1.3
Pancreas	ND	1.2 ± 0.8	0.1 ± 0.2*	0.7 ± 0.4
Submaxillary	ND	1.2 ± 0.3	ND	0.5 ± 0.1
Testis	ND	0.3 ± 0.1	0.3 ± 0.6*	1.2 ± 1.2
Lung	0.6 ± 0.7†	5.2 ± 1.2	3.5 ± 0.2	2.8 ± 1.4
Plasma	1.5 ± 0.4	12.2 ± 2.4	6.8 ± 2.2	32.4 ± 25.7

Tissue concentrations (μg/g wet tissue weight) were estimated 90 min after the i.v. administration of vitamin K (10 mg/kg). Warfarin-treated rats obtained *S*-warfarin (5 mg/kg) 16 hr before. Data are the means ± SD.

* Vitamin KO was detected in 1 out of 3; † Vitamin KO was detected in 2 out of 3; ND, not detectable.

warfarin binding might represent tissue vitamin K and/or vitamin KO reductase. Support for this is given by the observation that tissue bound warfarin in non-hepatic tissue also resides in the microsomal fraction. In addition, the data from the HS rat plead for such a relation with the target enzyme; in conformity with the mechanism of resistance, which involves a more rapid enzyme reactivation hence a faster release of the bound warfarin [11], tissue warfarin clearance was higher. Evidently, the rate of displacement by unlabeled warfarin was higher. Moreover, in conformity with the mode of action of the 4-hydroxycoumarins [12], the microsomal bound warfarin only dissociated substantially in the presence of large amounts of DTT. Liver microsomes of the HS rat release completely their warfarin at low DTT concentrations [11]. Remarkably, the enantiomeric composition of warfarin in liver microsomes 70 hr after the administration of racemic [^{14}C]warfarin was still racemic (Fig. 1), notwithstanding the fact that the *in vivo* rate of reactivation of the *R*-warfarin–enzyme complex is three to four times higher than that of the *S*-warfarin–enzyme complex [12]. It appears that the rate of trapping by the oxidized enzyme is much higher than the rate of escape from the intracellular target compartment. The dialysis experiment (Fig. 1) affirms the enantioselective differences in the reductive release of enzyme bound warfarin [12].

For those tissues tested, the *in-vitro* microsomal warfarin binding capacity parallels the *in-vivo* tissue distribution (Table 4). The microsomal vitamin KO reductase activity, however, deviates; for instance, pancreas reductase activity is low in comparison to the binding capacity. This may indicate the presence in these tissues of warfarin binding sites not related to vitamin KO reductase. However, it may also be true that the vitamin KO reductase assay which is optimalized for liver microsomes is not optimal for the other tissues.

We believe the data on warfarin distribution to reflect tissue distribution of the vitamin K cycle, at least that of vitamin K or vitamin KO reductase. The central question which needs to be answered is, what is the possible physiological function? The only function that we are aware of as yet is to provide the vitamin K-dependent carboxylase with vitamin K hydroquinone cofactor. This has been established for liver and bone, which produce Gla-containing coagulation and anticoagulation factors (liver), and bone Gla protein and matrix Gla protein (bone) (for reviews, see Refs 8 and 27). Not yet clarified, but probably present is the same function in kidney (matrix Gla protein [17]), lung (matrix Gla protein [17]; Gla-containing surfactant protein [28]) and aorta (plaque Gla protein [29]; protein S from endothelial cells [30]). Matrix Gla protein, i.e. its mRNA and immunoreactive protein, has also been identified in heart, spleen and brain tissue [17]. However, as evident from the study, heart and brain tissues do not posses the warfarin sensitive reductase activity.

Some tissues like testis and skin did not displace the accumulated warfarin. This does not necessarily mean that these tissues contain non-selective or non-saturable warfarin binding; for instance, several papers have reported on testicular vitamin KO reductase activity and this study showed the presence of warfarin binding sites in testicular microsomes. For warfarin to dissociate from its binding with vitamin KO reductase, the enzyme has to be reactivated by the endogenous reductant [12]. Thus, a reason for the non- or weak displaceability may be the absence of endogenous reductant activity; in other words the vitamin K cycle is not or only weakly operating.

Contrary to expectations, however, kidney, pancreas, and submaxillaris in warfarin-treated rats did not accumulate vitamin KO to any appreciable extent (Table 5). Either vitamin KO formation in these tissues is absent (in the presence of warfarin?) or vitamin KO elimination routes others than via vitamin KO reductase are present. For the formation of vitamin KO the reduction of vitamin K to the hydroquinone is needed. A microsomal NADH-dependent vitamin K reductase bypasses the warfarin block of the dithiol-dependent reductase in liver [31]. Possibly, these particular tissues have low activities of this NADH-dependent reductase as was shown for osteosarcoma UMR 106 cells [32].

The high level of warfarin binding sites in pancreas (about 70% of the level in liver) and salivary glands

(about 20% of the level in liver) is remarkable. Moreover, the rate of release of the bound warfarin is relatively high, indicating a high metabolic activity of the vitamin K cycle. As yet it is speculative as to whether this is related to their exocrine function.

In summary, this study provides evidence for the presence of an operative vitamin K cycle, i.e. the enzyme vitamin K and/or vitamin KO reductase, in various functional non-hepatic tissues. In particular, the high levels found in pancreas tissue are remarkable. Knowledge of the biochemical function of the extra-hepatic vitamin K cycle is needed to establish its physiological importance in tissues and to foresee potential adverse reactions of oral anticoagulants.

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