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Warfarin Binding to Microsomes Isolated from Normal and Warfarin-Resistant Rat Liver

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SUMMARY

A warfarin-binding site which had previously been demonstrated in liver microsomal membranes was studied. Although the binding site displayed apparently typical saturation binding curves when incubated with radioactive warfarin, the bound warfarin was not readily displaced by incubation with unlabeled warfarin. Warfarin-binding capacity was increased in membranes isolated from vitamin K-deficient rats, but was returned to normal when deficient rats were treated with 1 mg of phylloquinone or its chloro analogue. Prior treatment of animals with nonradioactive warfarin greatly decreased the subsequent binding capacity of the microsomal membranes *in vitro*. The warfarin-binding capacity was also very low in microsomes isolated from a strain of warfarin-resistant rats. The majority of the warfarin associated with the microsomal membranes isolated from warfarin-resistant rats was either bound very loosely or entrapped in vesicles, or it could be removed by phospholipase treatment. However, the warfarin associated with microsomal membranes from normal rats was bound to other membrane components, presumably protein.

INTRODUCTION

Strains of wild rats have been discovered in Scotland, Wales, and Denmark that are resistant to the vitamin K antagonist warfarin [3-(α-acetonylbenzyl)-4-hydroxycoumarin] and other coumarin anticoagulants. This trait is inherited by an autosomal dominant gene (1, 2). Homozygous, warfarin-resistant rats have been found not only to be 50–500 times less susceptible to repeated warfarin feedings than normal rats, but also to require 20 times more phylloquinone (1). Warfarin resistance is unrelated to the ability of these rats to metabolize warfarin (2) or vitamin K (3). It has therefore been suggested that the mutation is one that alters a regulatory protein required for synthesis of the vitamin K-dependent clotting factors such that the affinity for vitamin K is lowered only slightly while the affinity for warfarin is diminished greatly (1).

The site of synthesis of prothrombin and the other vitamin K-dependent clotting factors has been shown to be the liver (4). When subcellular fractions were prepared (3) from normal and warfarin-resistant rats treated with injections of radioactive warfarin, the microsomal fraction, but no other fraction, was found to contain more radioactivity. When ribosomes were isolated from liver microsomes that had been incubated in radioactive warfarin, ribosomes from war-

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farin-resistant rats had only 20–30 % as much warfarin bound to them as did ribosomes from normal rats (3). This difference in binding was hypothesized to be due to the presence of the altered warfarin-binding protein in the ribosomal preparations from resistant rats. This specific binding was found only with liver ribosomes, since ribosomes isolated from kidney, heart, and spleen exhibited low, nonspecific binding whether prepared from normal or warfarin-resistant rats.

These studies did not determine whether the radioactivity associated with the ribosomes was actually bound to the ribosomes or was due to the presence of warfarin bound to microsomal membranes which cosedimented with the ribosomes during their preparation. The present study was undertaken to determine the actual site of warfarin binding in liver microsomes and to determine the properties of warfarin binding to liver microsomes *in situ*.

MATERIALS AND METHODS

Drugs. Phylloquinone (AquaMephyton) was obtained from Merck Sharp & Dohme. 2 - Chloro - 3 - phytyl - 1,4 - napthoquinone (Chloro-K) was donated by Dr. J. Lowenthal, McGill University, Montreal, and was dissolved in 0.9 % NaCl with 7 % Tween 80. Warfarin sodium and [4-^{14}C] warfarin (25 mCi/mmole) were donated by Dr. K. P. Link, University of Wisconsin. The radioactive warfarin migrated as a single spot in a solvent system composed of *tert*-butyl alcohol–benzene–concentrated NH_4OH–H_2O (45:20:9:3). Phenobarbital (USP, J. T. Baker Chemical Company) was dissolved in 0.1 N NaOH to give a solution containing 16 mg of phenobarbital per milliliter; the solution was adjusted to pH 8.5 with concentrated HCl.

Treatment of animals. Normal, male Holtzman rats and male rats homozygous for the warfarin-resistant trait (1) were used. When vitamin K-deficient rats were required, they were housed for 7 days in coprophagy-preventing cages (5), and were fed a diet low in vitamin K (6). Phylloquinone and its chloro analogue were injected intramuscularly at a dose of 1 mg/rat. Warfarin (5 mg/kg) was administered as the sodium salt in 0.9 % NaCl by intraperitoneal injection.

Experimental procedures. Animals (180–200 g, body weight) were routinely fasted for 16 hr and killed by decapitation at 8 a.m. The livers were quickly removed and chilled immediately in 0.25 M sucrose–TKM (0.05 M Tris-HCl, pH 7.5, at 20°; 0.025 M KCl; and 0.05 M $MgCl_2$). The livers were weighed, minced with scissors in 2 volumes of 0.25 M sucrose–TKM, and homogenized with 10 strokes at 1700–1800 rpm in a Potter-Elvehjem homogenizer with a motor-driven pestle (clearance, 0.01 inch). The homogenate was centrifuged for 10 min at 17,000 × g_{max} to obtain the postmitochondrial supernatant fraction. Microsomes were prepared by taking 2 ml of the postmitochondrial supernatant fraction, adding 0.25 M sucrose–TKM to 10 ml, and centrifuging for 1 hr at 105,000 × g_{avg}. The results obtained with this preparation were similar to those with the bicarbonate-buffered preparation used previously (3). All procedures were carried out at 0–4°.

Microsomal membranes were prepared according to the discontinuous gradient method of Kashnig and Kasper (7). Microsomal membranes plus the upper 2.3 ml of each gradient were removed and diluted to 10 ml with TKM buffer. The membranes were pelleted by centrifuging for 35 min at 165,000 × g_{avg} and were resuspended in 0.25 M sucrose–TKM. Stripped ribosomes were obtained by taking the lower 2.3 ml of each gradient, diluting with TKM buffer to 10 ml, and centrifuging for 2 hr at 165,000 × g_{avg}. Ribosomal pellets were resuspended in TKM buffer.

Microsomal subfractions were isolated by the method of Dallner and Nilsson (8, 9), using a Spinco SW 50L rotor. Rough and total smooth microsomes were separated by centrifuging for 75 min at 204,000 × g_{avg}, while smooth microsomal fractions I and II were separated by centrifuging for 40 min at the same force. Each subfraction was resuspended in 0.25 M sucrose–TKM and used for warfarin binding studies.

Microsomes, at a total protein concentration of 5 mg/ml, were incubated in 2.5 μM [4-^{14}C] warfarin (10) for 1.5 hr in 0.25 M sucrose–TKM. The incubation was terminated, and the membranes were either removed from suspension by centrifugation, or

sodium deoxycholate was added to a final concentration of 0.25% (w/v) before the suspension was diluted to 10 ml with TKM and centrifuged for 10 min at 105,000 × g_{avg} to obtain deoxycholate-treated microsomal membranes. The resulting supernatant fluid was then centrifuged for 1 hr at 105,000 × g_{avg} to isolate deoxycholate-treated ribosomes. Each pellet was washed once and recentrifuged. Microsomal subfractions were incubated in the same manner, and stripped ribosomes were incubated as above, at a concentration of 0.5 mg of RNA per milliliter. The incubation was terminated, and the ribosomes were washed once.

Protein concentration was determined by a modification of the Folin phenol method (11) on suspensions of the various fractions. RNA was determined by a modified Schmidt-Thannhauser assay (12). Lipid was extracted by the method of Bligh and Dyer (13), and organic phosphorus was assayed in the chloroform layer by the method of Fiske and SubbaRow (14). Cytochrome P-450 was assayed as described by Omura and Sato (15).

Radioactivity was determined by dissolving 0.4-ml aliquots of the various fractions in 2 ml of an organic base ("NCS," Nuclear-Chicago) before addition of a toluene–2,5-diphenyloxazole–1,4 - bis[2 - (4-methyl-5-phenyloxazolyl)]benzene solution. A liquid scintillation spectrometer was used, and absolute counting rates were obtained by external standardization. The data were expressed as picomoles of warfarin bound, based on the specific activity (55.5 dpm/pmole) of the warfarin added.

RESULTS

The results of the chemical characterization of the preparations, and of the incubation of microsomal membranes and stripped ribosomes in radioactive warfarin, are shown in Table 1. The chemical analyses indicated that good separation of ribosomes and membranes had been achieved. Microsomal membranes from normal rats showed selective binding of warfarin, while stripped ribosomes showed only low, nonselective binding. Similar negative results were obtained when more highly purified ribosomes (16) were used in warfarin binding experiments. The ratio of warfarin binding in normal compared to warfarin-resistant microsomal membrane preparations varied from 1.5 to 2.5 in five experiments. These results indicate that the warfarin-binding site is located in microsomal membranes and not in the ribosomes, as suggested by the previous data (3).

Rough microsomes from normal and warfarin-resistant rats showed a high RNA content (RNA to protein ratio, 0.14), while smooth microsomal fractions I and II had a low RNA content (RNA to protein ratio, approximately 0.02 for both fractions). Table 2 shows the amount of warfarin bound to

TABLE 1

Chemical characterization and binding of warfarin to isolated microsomal membranes and ribosomes

Microsomal membranes and ribosomes were prepared from the pooled livers of four rats and incubated as described in the text. No differences in RNA to protein or phospholipid to protein ratios were found between normal and resistant rats, and the data from both groups have been pooled.

	RNA: protein	Phospholipid: protein	Warfarin bound		Binding ratio, N:R
			Normal (N)	Resistant (R)	
			pmoles/mg protein		
Membranes	0.01	0.56	42.0	17.7	2.4
Ribosomes	0.45	0.11	3.7	4.4	0.8

TABLE 2

Warfarin binding to microsomal subfractions

Microsomal fractions were prepared from the pooled livers of four rats and incubated as described in the text.

Microsome fraction	No detergent		Ratio, N:R	After detergent		Ratio, N:R
	Normal (N)	Resistant (R)		Normal	Resistant	
	pmoles warfarin/mg protein			*pmoles warfarin/mg protein*		
Smooth I	22	9	2.4	31	4	7.7
Smooth II	5	4	1.3	5	2	2.5
Rough	44	34	1.3	37	8	4.6

each microsomal subfraction after incubation in radioactive warfarin. Prior to the addition of detergent, only the smooth microsomes I from normal rats showed selective binding of warfarin; however, after addition of detergent, rough microsomes also showed a large degree of selective binding of warfarin. The difference was presumably due to the release of entrapped or nonselectively bound warfarin. Smooth microsomal fraction II bound very little warfarin either before or after detergent treatment. No more than 12% of the added warfarin was bound to the incubated microsomes or the various microsomal fractions.

Table 3 shows the effect of the vitamin K status of the animals and prior treatment with anticoagulants on the subsequent binding of warfarin *in vitro*. Nutritional vitamin K deficiency caused a 24% increase in the amount of warfarin which could be bound to deoxycholate-treated microsomal membranes. Treatment of the vitamin K-deficient animals with 1 mg of phylloquinone or its chloro analogue 1 hr before death returned warfarin binding to normal levels. Injection of warfarin 22 hr before the rats were killed caused a reduction of approximately 90% in warfarin binding *in vitro*. These results show that warfarin, or one of its metabolites, was tightly bound to microsomal membranes and was not removed during the fractionation procedure.

TABLE 3

Effect of vitamin K status and prior treatment with anticoagulants on warfarin binding in vitro

Phylloquinone (1 mg) or Chloro-K (1 mg) were given 1 hr, and warfarin (5 mg/kg) 22 hr, before the rats were killed. Vitamin K-deficient rats had 25%, and warfarin-treated rats 10%, of the normal prothrombin concentration when they were used. Phylloquinone treatment restored the prothrombin concentration to 60% of normal in 1 hr. Microsomes were prepared and incubated with radioactive warfarin as described in the text. Values are means ± standard errors for the numbers of animals indicated in parentheses.

Treatment	Specific activity
	pmoles/mg protein
Control (8)	93 ± 4
Deficient (8)	116 ± 5[a]
Deficient + phylloquinone (3)	86 ± 6[b]
Deficient + Chloro-K (3)	84 ± 6[b]
Warfarin (6)	10 ± 1[a]

[a] Significantly different at $p = 0.01$ from controls.

[b] Significantly different at $p = 0.01$ from deficient animals; not significantly different at $p = 0.05$ from controls.

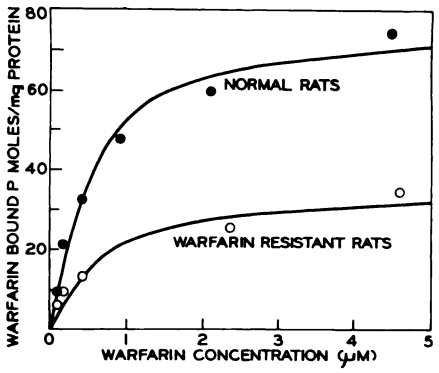

FIG. 1. *Effect of warfarin concentration on binding of warfarin to microsomal membranes*

Membranes from both types of rats were prepared as described in the text and incubated for 1.5 hr in the standard system. The points are the averages of values from two experiments, each utilizing pooled livers from four rats. The binding at each warfarin concentration did not differ more than 10% in the two experiments. Similar curves were seen when unfractionated microsomes were incubated and the membranes subsequently were isolated by deoxycholate treatment.

When microsomal membranes from both normal and warfarin-resistant rats were incubated in various concentrations of warfarin (Fig. 1), what appeared to be typical saturation binding curves were obtained. Similar binding curves were obtained when microsomes were first incubated in various concentrations of warfarin and then deoxycholate-treated microsomal membranes were isolated. To determine whether warfarin binding was reversible, microsomes were incubated in 2.5 μM radioactive warfarin for 1.5 hr, followed by the addition of a large excess of unlabeled warfarin. When the amount of labeled warfarin bound to the deoxycholate-treated microsomal membranes was examined, there was no significant exchange for up to 4 hr, even with a

1000-fold excess of unlabeled warfarin. Thus it appears that warfarin binding *in vitro* may be essentially irreversible, and that what appear to be binding curves may represent a slow titration of these irreversible sites by warfarin.

The amount of radioactive warfarin present at a very labile binding site, entrapped by microsomal membrane vesicles, or associated with phospholipid was determined by incubating the membrane preparations with buffer or with phospholipase C. Incubation with dilute buffer alone resulted in the loss of roughly the same amount of labeled warfarin from both normal and resistant rat microsomal preparations. This loss presumably represented the removal of warfarin from labile binding sites, or warfarin which was entrapped in the previously washed microsomes. Incubation of the preparations with phospholipase resulted in an additional loss of warfarin, which presumably was associated with a lipid component of the membrane, rather than being tightly bound to a membrane protein. These data are summarized in Table 4. The amount of total activity released by buffer and enzyme treatment was found to be about the same in the membrane preparations from both strains of rats. The specific activity of the preparation from normal rats was roughly 7 times that from resistant rats following this treatment. The results suggest that the strain-specific difference in warfarin binding was not due to permeability factors, or to the binding of warfarin to phospholipid. The decreased warfarin binding in microsomal membrane preparations from warfarin-resistant rats was presumably due to the presence of an altered warfarin-binding protein.

TABLE 4

Effect of buffer or phospholipase C treatment on warfarin binding to microsomal membranes

Five milligrams of microsomal membranes, labeled with warfarin, were incubated for 30 min at 37° in 2 ml of 0.04 M Tris buffer, pH 7.8, or in buffer with 10 mM $CaCl_2$ and 0.5 mg of phospholipase C added. After incubation, the samples were chilled, diluted with cold buffer, and centrifuged at 165,000 × *g* for 30 min.

Membrane fraction	Warfarin bound	
	Normal	Resistant
	pmoles/fraction	
I. Membranes before treatment	298	169
II. Membranes after buffer incubation	196	52
III. Membranes after enzyme treatment	140	19
IV. Warfarin released by buffer (calculated, I — II)	102	117
V. Warfarin released by enzyme (calculated, II — III)	56	33

Olson (17) has hypothesized that a regulatory protein which can bind both warfarin and vitamin K interacts with translocase, the cycloheximide-binding protein on the large subunit of mammalian ribosomes, and that cycloheximide is a partial competitive inhibitor of vitamin K. No significant difference in the amount of warfarin bound to deoxycholate-treated membranes was seen when 1.8 mM cycloheximide was included in the system.

Since many drugs (18) are known to bind to cytochrome P-450, it was considered possible that the warfarin-binding site in microsomal membranes could be cytochrome P-450. Phenobarbital was administered (19) to both strains of rats, and cytochrome P-450 was found (Table 5) to increase to 213% of control levels in normal animals and 237% of control levels in warfarin-resistant animals. Microsomal protein was found to be 147% of control levels in normal rats, but only 108% of control values in warfarin-resistant rats. Phenobarbital administration caused a 32% decrease in the specific activity of warfarin bound to deoxycholate-treated microsomal membranes, but did not affect warfarin binding in warfarin-resistant rats. These findings suggest that cytochrome P-450 did not bind warfarin and that warfarin binding to microsomal membranes was due to another component of the membranes.

DISCUSSION

The warfarin-binding site previously observed to be associated with crude preparations of ribosomes (3) has been found to be

TABLE 5

Effect of phenobarbital treatment on warfarin binding

Three animals of each group were given 40 mg/kg of phenobarbital intraperitoneally for 4 consecutive days and were killed 24 hr after the last injection. Cytochrome P-450 assays were performed on whole microsomes. Warfarin binding was assayed on deoxycholate-treated membranes after disruption of the microsomes with detergent. Values are means ± standard errors.

Rat strain	Microsomal protein		Cytochrome P-450		Warfarin binding/ mg protein	
	Control	Phenobarbital	Control	Phenobarbital	Control	Phenobarbital
	mg/g liver		*nmoles/g liver*		*pmoles/mg*	
Normal	23 ± 3	34 ± 2	14 ± 2	44 ± 3	101 ± 7	69 ± 3
Warfarin-resistant	26 ± 2	31 ± 3	16 ± 2	46 ± 1	18 ± 1	19 ± 1

localized in microsomal membranes. The ribosomal binding was presumably due to the presence of contaminating membrane components in the ribosomal preparations, which were also observed (20, 21) when crude ribosomes were prepared from microsomes by deoxycholate disruption. The lack of a specific warfarin-binding site in ribosomes was further demonstrated when stripped ribosomes were incubated in warfarin and no strain-specific binding was observed. These preliminary data indicate that the warfarin-binding site is present in both rough and smooth fraction I microsomes. The determination of the actual location and relative amount of the warfarin-binding site in the membranes of microsomal subfractions must, however, await the isolation of pure membranes from each subfraction.

These studies also demonstrate an increased binding of warfarin to deoxycholate-treated microsomal membranes prepared from vitamin K-deficient animals. This increased binding could have been due to one of two generalized effects. A warfarin-binding site could have been unmasked because vitamin K was removed from a common binding site, or through conformational changes of a protein, or proteins, caused by the removal of vitamin K from its binding site. Alternatively, the synthesis of the warfarin-binding protein could have been increased in vitamin K deficiency. The rapidity with which vitamin K blocked the increased binding in microsomal membranes from deficient animals seems to rule out the second possibility. The present experiments did not show whether the return to normal binding was due to conformational changes or whether vitamin K directly blocked warfarin-binding sites.

The chloro analogue of phylloquinone was also observed to block the increased warfarin binding in crude membranes from vitamin K-deficient rats. Lowenthal (22) has suggested that phylloquinone is transported in the cell via both a coumarin anticoagulant-sensitive transport route and, at higher levels, by an alternative, non-coumarin-sensitive route. He has proposed that chlorophylloquinone is transported only by the alternative route, which is insensitive to the coumarin anticoagulants. Both phylloquinone and its chloro analogue might then interact competitively with a receptor site that is not directly affected by the coumarin anticoagulants. The present finding of an interaction of chlorophylloquinone with a warfarin-binding site seems to contradict this hypothesis, and suggests that coumarin anticoagulants bind at the same site as vitamin K or to sites in close proximity. Attempts to block microsomal warfarin binding with vitamin K *in vitro*, or to obtain significant labeling of isolated microsomes (3) by incubation with radioactive phylloquinone, have not been successful. Whether or not this extremely insoluble lipophilic compound actually reaches the same membrane sites as the water-soluble warfarin is not known.

Warfarin binding to isolated microsomal membranes appeared to be essentially irreversible for the period investigated. The

binding curves that were obtained were typical of those seen for saturation binding, but because of the irreversible nature of the binding, these curves were interpreted to be titration curves for the binding of warfarin to apparently irreversible sites. A similar situation was seen *in vivo*. When the rats had been treated with warfarin, the binding sites were apparently saturated and would not subsequently exchange with radioactive warfarin. The available data do not offer a satisfactory explanation for this behavior. Pool *et al.* (2) have noted that factor VII production in liver slices from both normal and warfarin-resistant rats is blocked by warfarin. How this relates to the situation *in vivo* is not clear, as Pool and Borchgrevink (23) have also shown that warfarin blocks both factor VII production and protein synthesis in this system, but only factor VII production *in vivo*.

Because the amounts of warfarin released by buffer or phospholipase treatment of microsomal membranes from normal and warfarin-resistant rats were found to be the same, the lower amount of warfarin associated with microsomal membranes from warfarin-resistant rats must be due to the binding of less warfarin to a specific site on the protein. The activity which remained bound to the microsomes from warfarin-resistant rats was so low that it could not be determined whether the binding protein was completely missing or whether its affinity was altered. These observations generally support the hypothesis of Thierry, Hermodson, and Suttie (3) that an altered warfarin-binding protein exists in the livers of warfarin-resistant rats. Although cytochrome P-450 has been reported to bind warfarin (24), there was no indication that it was involved in this species-specific binding. The data do not, however, completely exclude the binding of warfarin to some neutral lipid component of the membrane. Determination of the exact nature of the warfarin-binding protein must await its solubilization and purification. Preliminary attempts to purify the protein have been unsuccessful. In general, procedures which have resulted in effective solubilization of the membrane have also caused a loss of binding.

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