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ACTION OF WARFARIN INJECTED INTO RATS ON PROTEIN SYNTHESIS *IN VITRO* BY LIVER MICROSOMES AS RELATED TO ITS ANTICOAGULATING ACTION

NAOMI BIEZUNSKI*

Department of Medicine, Harvard Medical School and Beth Israel Hospital, Boston, Mass., U.S.A.

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Abstract—Warfarin injected into rats inhibits protein synthesis *in vitro* by liver microsomes. The synthesis of all liver proteins, not only the prothrombin complex, is inhibited. This inhibition was found in 10 of 13 groups of animals and was not influenced by the origin of the cell sap, nor was it correlated with the degree of depression of prothrombin levels found in the plasma and in the microsomes. Vitamin K injected into rats after warfarin almost completely restored the level of the prothrombin complex in plasma and in microsomes; however, the inhibition of protein synthesis by liver microsomes was relieved only to a slight degree. The mechanism of the inhibition of protein synthesis *in vitro* in liver microsomes by warfarin injected into rats and the effect of vitamin K are discussed.

IT HAS long been known that coumarin (warfarin,† dicumarol‡) depresses plasma levels of prothrombin, Factor VII and Factor X (prothrombin complex) as measured by their biological activity. Although the levels of other plasma proteins are not depressed by the drug, Martius and Nitz-Litzow[1] and Martius,[2] who found that dicumarol uncouples oxidative phosphorylation, suggested a general inhibition of protein synthesis. The observable effect on prothrombin levels was attributed by these investigators to the rapid turnover of its proteins. Subsequent work failed to confirm this assumption. Green *et al.*[3] reported that mitochondria prepared from dicumarol-treated rats show normal oxidative phosphorylation. However, when dicumarol is added *in vitro* to normal liver mitochondria, it depresses oxidative phosphorylation, and vitamin K is unable to reverse the uncoupling effect of dicumarol. Paolucci *et al.*[4] confirmed this finding *in vitro* and also reported that normal oxidative phosphorylation occurs in mitochondria derived from rats with severe vitamin K deficiency. Hill *et al.*[5] found that vitamin K deficiency has no effect on protein synthesis *in vivo* and in liver microsomes *in vitro*. Couri and Wosilait,[6] using as an index the content of adenine nucleotides and ^{32}P-orthophosphate incorporation into liver nucleotides in intact rats, found that coumarin drugs have no effect on oxidative phosphorylation *in vivo*.

In addition to their anticoagulant effect, the coumarin drugs have pharmacological

* Present address: Department of Virology, Hebrew University—Hadassah Medical School, Jerusalem, Israel.

† 3-(a-Acetonylbenzyl)-4-hydroxycoumarin.

‡ 4,4′-Dihydroxy-3,3′-methylene biscoumarin.

actions that are unrelated to coagulation. These are: depression of cardiac muscle and smooth muscle contractility, vasodilatation, uricosuria and antibacterial action.[6,7] Further, reduced oxygen consumption relative to lipid peroxidation was found in microsomes from vitamin K-deficient rats and rats treated with dicumarol or warfarin, and there was no correlation with lengthening of prothrombin time.[8]

The present communication reports a lower amino acid incorporating activity by liver microsomes from warfarin-injected rats as compared to normal rats. The depression was observed in most preparations and this effect seems to be independent of the depression of prothrombin levels regularly produced by the drug.

MATERIALS AND METHODS

Injection of animals and preparation of incubation mixture. Male 150 g Charles River CD rats were fed Purina chow *ad lib.*, before and during the experiments. Groups of two to three rats were injected with sodium warfarin (Coumadin from Endo Laboratories Inc.) with or without vitamin K_1 (Aquamephyton from Merck, Sharpe & Dohme). Controls received 0·9% NaCl. After 17–48 hr, animals were decapitated. Liver microsomes and the incubation mixtures were prepared by the technique of Williams *et al.*[9] The livers of three rats from each group were suspended in 2·5 v/w of ice-cold solution containing 0·01 M $MgCl_2$, 0·035 M Tris (pH 7·8) and 0·25 M sucrose. The livers were cut into small pieces and homogenized in a glass Potter–Elvehjem homogenizer with a smooth Teflon pestle. Samples were homogenized at 4° and during homogenization the pestle was raised and lowered six times. After centrifuging the homogenates twice at 15,000 *g* for 10 min, the upper two-thirds of the supernatant was collected and centrifuged at 105,000 *g* for 90 min. The supernatant fluid was collected and used as the supernatant fraction in the incubation mixture. The microsomal pellet was resuspended by gentle homogenization in 0·25 vol. of the same buffer solution and used as the microsomal fraction. The protein content and the RNA content of these fractions were estimated. A 2·6 mg portion of protein of the supernatant fraction and 3·4 mg protein of the microsomal fraction from normal or treated rats per milliliter of incubation mixture were used. Although identical amounts of protein of microsomal and cell-sap fractions were used in the incubation mixtures, occasionally warfarin-treated rats had reduced liver weights. The ratio of protein to RNA in these livers was the same as that in normal rats. Where there was liver weight loss, a lower yield of microsomes was obtained, but most warfarin-treated animals gave yields comparable to those of the control groups. The loss of liver protein did not appear to be correlated with inhibition of either plasma prothrombin activity or protein synthesis *in vitro*. In addition to liver fractions, the incubation mixture contained: 0·4 mM GTP; 7·4 mM glutathione; 2 mM ATP; 8 mM phosphoenolpyruvate (PEP); 40 μg PEP-kinase per ml of incubation mixture; 56 mM KCl; 25 mM Tris (pH 7·8); 6 mM $MgCl_2$; 0·1 M sucrose; 4 μg polyvinylsulfate per ml of incubation mixture. In some experiments 6 μc of ^{14}C-amino acids per ml of incubation mixture was added in the form of yeast hydrolysate (Schwarz BioResearch, Inc.; 6 μc = 4·8 μg). In others, 15 μc ^{3}H-L-leucine (1–5 c/m-mole; Nuclear-Chicago) per ml of incubation mixture was added.

Radioactivity determination. The mixture was incubated at 37° for 50 min; samples were taken, at the start of and during incubation, into cold 5 per cent trichloroacetic acid containing 0·1 per cent of unlabeled casein hydrolysate or, when ^{3}H-L-leucine was

used, 1 mM unlabeled L-leucine. The precipitate was washed once with the above solution, heated for 15 min at 90°, cooled and washed once more with 5 per cent trichloroacetic acid. Samples with ^{14}C-label were passed through Millipore filters and the filters were counted in a thin-window gas flow Nuclear-Chicago counter; the ^{3}H-L-leucine-labeled samples were dried at 110°, dissolved in 5 drops of formic acid and counted in a Nuclear-Chicago scintillation counter using the following scintillation solution: 2 ml methanol and 10 ml toluene containing 6 g PPO* and 0·6 g POPOP*/l.

Prothrombin activity assay. Prothrombin activity was measured by the two-stage method of Ware and Seegers,[10] with the modifications introduced by Goldstein *et al.*[11] Oxalated plasma from normal rats was used as the control. One ml blood from each decapitated rat was placed in a plastic tube containing 0·1 ml of 0·1 M sodium oxalate. For the determination of prothrombin activity in liver fractions, this technique was modified as follows: 0·1 vol. of 0·1 M sodium oxalate was added to undiluted samples of liver fractions or incubation mixture fractions. Factors V and VII were used at 10 times the concentration in $\frac{1}{10}$ of the usual volume.

Fractionation of incubation mixture. After 50 min of incubation, the microsomes were separated from the incubation mixture by centrifugation and fractionated by the method of Helgeland.[12] Further fractionation of the supernatant of the incubation mixture and microsomes fractions for purification of prothrombin was done according to the method of Goldstein *et al.*[11] by barium sulfate adsorption. The method of Lowry *et al.*[13] was used for estimation of protein. The amount of RNA in hot trichloroacetic acid extracts of liver fractions was estimated by the orcinol method.

RESULTS

Figure 1 shows the pattern of inhibition of protein synthesis in incubation mixtures (liver microsomes and cell sap) prepared from warfarin-treated rats. It is evident that both the initial rate and the extent of amino acid incorporation were inhibited as compared with the control.

Table 1 presents data on protein synthesis in liver microsomes prepared from rats

TABLE 1. INCORPORATION OF AMINO ACIDS BY LIVER MICROSOMES FROM NORMAL SALINE-INJECTED RATS AND RATS INJECTED WITH WARFARIN AND WARFARIN + VITAMIN K*

Incubation time (min)	Rats injected/100 g body wt., 39 hr before decapitation		
	Normal saline	Warfarin (250 μg)	Warfarin (250 μg) + vitamin K (1 mg)†
5	0·9	0·58	0·8
10	1·7	1·0	1·2
15	2·1	1·1	1·4
30	2·0	1·3	1·6

* Numbers represent counts/min × 10^{-4}/0·25-ml sample of incubation mixture.

† Vitamin K was injected 13 hr before decapitation. The incubation mixture was prepared as described in the legend to Fig. 1. ^{14}C-yeast hydrolysate was used.

* PPO = 2,5-diphenyloxazole; POPOP = 1,4-bis-2-(4-methyl-5-phenyloxazolyl)benzene.

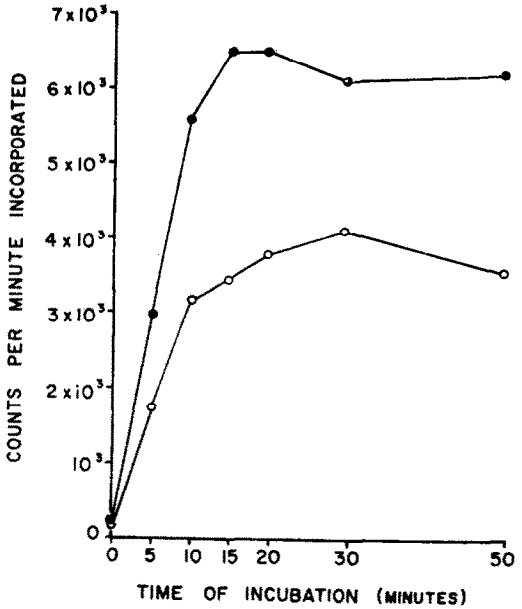

FIG. 1. Protein synthesis by liver microsomes from normal saline-injected and warfarin-injected rats: ●—●—●, normal saline-injected rats; ○—○—○, rats injected i.v. with warfarin (250 μg/100 g) 39 hr previously. The incubation mixture was prepared as described in Methods; 6 μc ^{14}C-yeast hydrolysate per ml of incubation mixture was used. Microsomes and supernatant were prepared from the pooled livers of three rats of each group.

TABLE 2. PROTHROMBIN ACTIVITY IN LIVER MICROSOMES FROM NORMAL SALINE-INJECTED RATS AND RATS INJECTED WITH WARFARIN, WARFARIN + VITAMIN K, AND VITAMIN K*

Rats injected/100 g body wt.	Prothrombin activity	
	(units/ml)	(units/g protein)
Normal saline	1·6	67
Warfarin (250 μg)	0	0
Warfarin (250 μg) + vitamin K (1 mg)	1·0	36
Vitamin K (1 mg)	1·35	55

* Rats were injected 39 hr before decapitation, except for vitamin K, which was injected 13 hr before decapitation. Prothrombin activity was measured in microsomes pooled from two livers of each group, as described in Methods.

injected with normal saline, warfarin, or warfarin plus vitamin K. Injection of warfarin reduced the prothrombin activity in the plasma of rats to 5–12 per cent of the control level, and inhibited protein synthesis *in vitro* by liver microsomes. Injection of vitamin K restored the prothrombin activity to 85 per cent of the level of the control, but only partially corrected the protein synthesis. Table 2 shows the prothrombin activity in liver microsomes from variously treated rats. Prothrombin activity was similar in rats injected with normal saline or vitamin K, but it was unmeasurable in

microsomes from warfarin-treated rats. Injection of vitamin K, 1 mg/100 g body wt., 26 hr after warfarin and 13 hr before decapitation, restored the prothrombin activity of the microsomes. These results paralleled the effect of warfarin and vitamin K on plasma prothrombin activity.

Protein synthesis in an incubation mixture prepared with microsomes from warfarin-treated rats and cell sap from normal saline-injected rats was inhibited to the same degree as the protein synthesis in an incubation mixture prepared wholly from warfarin-injected rats (Table 3).

Table 4 summarizes the effect of warfarin on amino acid incorporation by liver

TABLE 3. INCORPORATION OF AMINO ACIDS BY LIVER MICROSOMES FROM NORMAL SALINE-INJECTED OR WARFARIN-INJECTED RATS AND IN A MIXED INCUBATION MIXTURE*

Incubation time (min)	Rats injected 24 hr before decapitation		
	Normal saline	Warfarin (250 μg/100 g body wt.)	Mixed incubation mixture†
5	2·5	1·5	1·7
10	5·0	3·0	3·0
15	6·0	4·3	4·2
30	6·5	4·9	4·7

* Numbers represent cpm $\times 10^{-3}$/0·25 ml of incubation mixture. ^{14}C-yeast hydrolysate was used.

† The mixture contained microsomes from warfarin-injected rats and cell sap from normal saline-injected rats.

TABLE 4. EFFECT OF INJECTED WARFARIN ON PROTEIN SYNTHESIS BY RAT LIVER MICROSOMES *IN VITRO* AND ON PLASMA PROTHROMBIN ACTIVITY*

Expt. no.	Warfarin (μg/100 g)		Vitamin K (μg/100 g)	Interval from injection to decapitation (hr)	Inhibition of extent of protein synthesis† (% control)	Plasma prothrombin activity (% control)
1	100			17	50	24‡
2	50			17	50	
	100			17	50	67‡
	250			17	40	67‡
3	250			24	30	
	250	+	40	24	20	
4	250			39	40	5 12
	250	+	1000§	39	20	85 85
5	250			41	0	12 12
6	250			41	60	50 60
7	250			46	0	20 25 50
8	250			46	20	10 10 12
	500			46	0	13 16 17

* Protein synthesis and plasma prothrombin activity were compared with values of control, normal saline-injected rats.

† The values shown are rounded off to the nearest 5 per cent. Conditions of incubation are described in Fig. 1. Incubation time, 50 min.

‡ Prothrombin activity in these samples was determined in blood from rats injected with indicated amounts of warfarin, but not used for preparation of microsomes. All injections were intravenous, except in experiment 1, when rats were injected intraperitoneally. ^{14}C-yeast hydrolysate was used in experiments 1–4, and ^{3}H-L-leucine in experiments 5–8.

§ Vitamin K given 13 hr before decapitation.

microsomes and on plasma prothrombin activity in 13 groups of animals. In 10 of the 13 groups there was a marked inhibition (20–60 per cent) in amino acid incorporating activity. Plasma prothrombin activity was low in all groups of rats injected with warfarin, including those in which protein synthesis was unaffected.

In order to examine the possibility that inhibition of protein synthesis by warfarin does not affect all liver proteins, incubation mixtures derived from groups of normal saline-treated or warfarin-injected rats were fractionated after 50 min of incubation and the specific radioactivity and prothrombin activity of various fractions were estimated. The results are summarized in Tables 5–7. The results show reduced

TABLE 5. SPECIFIC RADIOACTIVITY OF PROTEINS SYNTHESIZED *IN VITRO* BY LIVER MICROSOMES FROM NORMAL SALINE-INJECTED AND WARFARIN-INJECTED RATS*

Rats injected	Specific radioactivity of proteins in fraction:				
	Supernatant of incubation mixture	Microsomes	Supernatant of sonicated microsomes	Supernatant of DOC-treated microsomes	Ribosomes
Normal saline	0·54	3·3	1·4	1·2	4·4
Warfarin (250 μg)	0·21	2·5	0·7	0·65	3·7

* Incubation mixtures prepared as described in Methods and incubated for 50 min were fractionated according to Helgeland[12] and the specific radioactivity of proteins in various fractions was estimated: two incubation mixtures were prepared from a pool of livers of three normal saline-injected rats and two from a pool of three rats injected with warfarin, 250 μg/100 g of body wt. Numbers represent dpm $\times 10^{-5}$/mg protein and an average of two incubation mixtures. ^{3}H-L-leucine was used. Rats were injected 46 hr before decapitation. DOC = deoxycholate.

TABLE 6. SPECIFIC RADIOACTIVITY OF PROTEINS IN FRACTIONS TREATED WITH BARIUM SULFATE*

Rats injected	Supernatant of incubation mixture		Supernatant of sonicated microsomes	
	Adsorbed by $BaSO_4$	Not adsorbed by $BaSO_4$	Adsorbed by $BaSO_4$	Not adsorbed by $BaSO_4$
Normal saline	0·82	0·2	2·5	2·1
Warfarin (250 μg)	0·43	0·06	1·6	0·75

* Fractions described in Table 5 were further fractionated by $BaSO_4$, a method used for purification of prothrombin,[11] and the specific radioactivity of protein fractions was estimated. In this procedure[11] the prothrombin complex proteins are adsorbed by $BaSO_4$. Numbers represent dpm $\times 10^{-5}$/mg protein and an average of two incubation mixtures.

TABLE 7. PROTHROMBIN ACTIVITY IN BARIUM SULFATE-ADSORBED FRACTION FROM SONICATED MICROSOMES OF NORMAL SALINE-INJECTED AND WARFARIN-INJECTED RATS*

Incubation mixture	Rats injected	Prothrombin		
		Time (sec)	(units/ml)	(units/g protein)
1	Normal saline	110	0·2	6000
2	Normal saline	85	0·3	6600
3	Warfarin (250 μg)	205	<0·1	
4	Warfarin (250 μg)	285	<0·1	

* Prothrombin activity was assayed as described in Methods. One ml of bovine plasma fractionated by $BaSO_4$ used as control gave 250 units/ml of prothrombin activity.

specific radioactivity of all liver proteins synthesized in the incubation mixture, prepared from warfarin-treated rats, in which protein synthesis was inhibited. The barium sulfate adsorption procedure[11] was used for the purification of prothrombin from the incubation mixture; it was found that only the microsomes and the $BaSO_4$-adsorbed fraction from sonicated microsomes contained prothrombin activity. The prothrombin activity of these fractions derived from warfarin-treated rats was considerably reduced as compared to the control. In incubation mixtures (see Table 4, expt. 8) from warfarin-injected rats in which protein synthesis was not inhibited, the specific radioactivity of all proteins was normal, in spite of the greatly reduced prothrombin activity of liver fractions.

DISCUSSION

The results of these studies show inhibition of protein synthesis *in vitro* by liver microsomes from warfarin-treated rats. No correlation between the effect of warfarin on prothrombin activity and on protein synthesis was observed. The inhibition of protein synthesis seemed to be more pronounced on the first day after injection of the drug, whereas plasma prothrombin activity dropped on the second day. The inhibition was found to be due to microsomes from warfarin-treated rats and was not influenced by the origin of the cell sap. When the specific radioactivity of proteins synthesized *in vitro* by liver microsomes from warfarin-treated rats was measured, it was found that the inhibition of protein synthesis by warfarin affects all liver proteins regardless of their prothrombin activity content.

The mode of action of warfarin inhibition is not clear. Inhibition of protein synthesis *in vitro* by dicumarol and warfarin in liver slices has been reported by several investigators.[6,14] On the other hand, no effect of dicumarol on protein synthesis *in vitro* by liver microsomes was found by Couri and Wosilait.[6] An important difference between our experiments and the work of Couri and Wosilait is that we injected whole animals with warfarin and tested the microsomes for their amino acid incorporating activity *in vitro*, whereas they treated the microsomes with the drug *in vitro* and found no effect on amino acid incorporation, but found inhibition when the drug was added to liver slices. It is possible that coumarin drugs act through the production of an active derivative *in vivo* and this can still be produced in liver slices when the drug is added, but not in microsomes.

Since 3 of 13 groups of animals showed no inhibition, it is possible that, when they were tested for their protein-synthesizing activity, the effect of warfarin had already disappeared or that the warfarin was leached out of these preparations. It is of interest that Couri and Wosilait[6] also suggested that the negative effect of dicumarol injected into rats on protein synthesis *in vitro* in liver slices may be due to leaching out of the dicumarol. Polson and Wosilait[15] observed a variable response of rats to puromycin on the prolongation of prothrombin time and it is possible that a response of rats to warfarin could also be variable. Couri and Wosilait[6] reported normal protein synthesis in liver microsomes from dicumarol-treated rats, but no experimental data were given. These results could also be explained as above, by loss of bound warfarin, by varying susceptibility to warfarin, and by varying time of warfarin effect.

Vitamin K injected after warfarin restored the prothrombin activity in plasma and microsomes, but only partially relieved the inhibition of protein synthesis. However, the mode of action of vitamin K in these systems is not well understood. Hill *et al.*[5]

found that the site of action of vitamin K on prothrombin activity is beyond the blocking of protein synthesis by cycloheximide, and they suggest that it acts in the removal of a properly folded precursor peptide of prothrombin.

The lack of correlation in the effect of warfarin on prothrombin activity and protein synthesis reported in this study, as well as the lack of effect of vitamin K deficiency on protein synthesis reported by others,[5] may suggest that different mechanisms are involved in the action of these two drugs on the two systems.

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