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INHIBITION OF VITAMIN K EPOXIDASE BY TWO NON-COUMARIN ANTICOAGULANTS

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Abstract—Tetrachloro-4-pyridinol (4-TCP) and 2-chloro-3-phytyl-1,4-naphthoquinone (Cl-K), like the coumarin and indanedione anticoagulants, caused the accumulation of prothrombin precursor activity in liver microsomes. Unlike the coumarins and indanediones, 4-TCP and Cl-K interrupted the vitamin K_1 vitamin K_1 epoxide cycle by inhibiting the epoxidation of vitamin K_1. Epoxidase activity assayed *in vitro* was decreased by about 40 per cent relative to controls in rats treated 24 hr previously with 4-TCP or Cl-K. *In vitro* assays demonstrated that 3×10^{-6} M Cl-K and 10^{-4} M 4-TCP inhibited the epoxidation of vitamin K_1 by about 75 per cent. Inhibition of phylloquinone epoxidase activity was determined *in vivo* by blocking the reduction of epoxide with warfarin and measuring the conversion of [^{3}H]K_1 to [^{3}H]epoxide. Doses of Cl-K and 4-TCP which blocked prothrombin synthesis also inhibited epoxidation while doses which did not lower plasma prothrombin also had no significant effect on the K_1 epoxide conversion.

When inhibition of prothrombin synthesis by 4-TCP and Cl-K was reversed by vitamin K_1, a minimum of 6–17 nmole of epoxide were formed in the liver for each nmole of prothrombin that appeared in plasma. The results suggest that epoxidation of vitamin K may be involved in prothrombin production and that interference with either the epoxidation or reduction step in the cycle will result in inhibition of clotting protein synthesis.

A major pathway of vitamin K metabolism in man and the rat is conversion to the 2,3-epoxide which is reduced back to the vitamin [1–3]. A number of observations have suggested that the vitamin K_1 vitamin K_1 epoxide cycle is involved in the mechanism of action of the vitamin and its antagonism by anticoagulants [4–6]. Coumarin and indanedione anticoagulants inhibit the reduction of the epoxide to the vitamin while two other anticoagulants, tetrachloro-4-pyridinol (4-TCP) and 3-phytyl-1,4-naphthoquinone (Cl-K) have little effect on this reaction [7]. Therefore, we investigated the possibility that 4-TCP and Cl-K may interrupt the cycle by inhibiting the epoxidation of vitamin K. We also wished to determine whether 4-TCP caused the accumulation of prothrombin precursor activity in the microsomes like warfarin and Cl-K [8].

MATERIALS AND METHODS

Materials. Male Sprague–Dawley rats (6–12 weeks old) fed Purina Laboratory Chow were used in these studies. [^{14}C]phylloquinone, uniformly labelled in the phytyl side chain, and 6,7-[^{3}H]phylloquinone were synthesized and purified as described previously [1, 9]. Cl-K was provided by J. Lowenthal (Pharmacology Department, McGill University, Montreal, Canada) and was purified by chromatography on silicic acid [1]. The sodium salts of 4-TCP and tetrachloro-2-pyridinol were gifts from F. Marshall (Dow Chemical Company, Zionsville, Ind.) and sodium warfarin from Endo Laboratories (Garden City, N.Y.). For intracardial injections (i.c.), [^{3}H]phylloquinone and Cl-K were dissolved in Tween 80 and diluted with 0.9% NaCl to make solutions containing 5% Tween or less. Sodium warfarin and sodium TCP were dissolved in water for intraperitoneal (i.p.) injections.

Epoxidase assay. Livers were prepared and assayed for phylloquinone epoxidase as previously described [10]. Rats were decapitated and the livers quickly excised and chilled. The livers were minced and homogenized using a Polytro 20ST homogenizer (Brinkmann Instruments) at low speed. Homogenates (25% w/v) were prepared in 0.25 M sucrose containing 0.05 M potassium phosphate buffer, pH 7.5. Homogenates were centrifuged at 15,000 *g* for 20 min in a Sorvall RC2-B centrifuge for preparation of supernatant fractions used in the epoxidase assay.

1.6 nmole of [^{14}C]phylloquinone (s.a. 70 mCi/mmole) dissolved in 50 μl of ethanol were added to incubation mixtures containing 1.5 ml of the liver supernatant, 30 nmole of sodium warfarin to inhibit vitamin K epoxide reductase [6], and homogenization solution to a final volume of 3 ml. The mixtures were shaken in open flasks at 37° in a Dubnoff incubator and the reaction terminated by the addition of 7 ml of isopropanol–hexane (3:2). The mixture was transferred to stoppered tubes, shaken vigorously and centrifuged at 1500 rev/min for 10 min. The extract, which contained 80–100 per cent of the added radioactivity, was separated by t.l.c. with carrier phylloquinone and phylloquinone epoxide as previously described [1, 5]. The phylloquinone and epoxide spots accounted for 90–100 per cent of the chromatographed ^{14}C.

Prothrombin precursor assay. The venom of *Echis carinatus* (Sigma Chemical Co., St. Louis, Mo.) has been used to generate thrombin activity from prothrombin [11] and from microsomal extracts of livers from hypoprothrombinemic rats [8]. Microsomes were prepared from 15,000 *g* liver supernatant by cen-

trifugation at 105,000 *g* for 1 hr. The microsomal pellet was suspended in fresh homogenizing medium at one-half the volume of the 15,000 *g* supernatant, treated with an equal volume of 1% Triton X-100 (Rohm and Hass, Philadelphia, Pa.) and dialyzed for 18–20 hr against 0.025 M potassium phosphate buffer, pH 7.5. Insoluble material was removed by centrifugation at 105,000 *g* for 1 hr. In order to remove any prothrombin, the supernatant was treated with $BaSO_4$ (30 mg/ml) for 1 hr with frequent mixing in an ice-bath. The $BaSO_4$ was removed by centrifugation at 2000 *g* for 5 min. A portion of the supernatant (0.2 ml) was incubated with 0.1 ml of *Echis carinatus* venom in water (1 mg/ml) at 37° for 20 min. The timed clotting reaction was started by adding 0.1 ml of $BaSO_4$ absorbed beef plasma [12] and measured with a coagulation timer (Fibrometer clot timer, BBL, Cockeysville, Md.). No clot was observed if beef plasma was incubated with microsomal protein or snake venom alone. The clotting times obtained were converted to NIH thrombin units by comparison with a standard curve prepared by dilution of a standardized solution of thrombin (Thrombin, Topical; Parke-Davis, Detroit, Mich.).

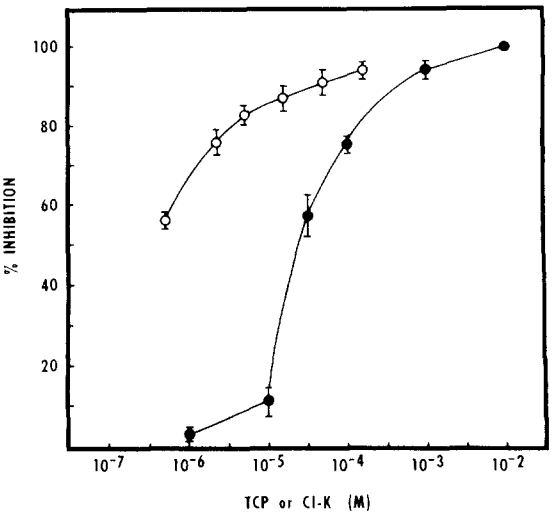

Fig. 1. Inhibition of phylloquinone epoxidase *in vitro* by Cl-K (○) and TCP (●). 4-TCP, dissolved in homogenizing medium, or Cl-K, dissolved in 50 μl ethanol, were added to incubation mixtures (see Materials and Methods). Each point represents the average ± S.E.M. of triplicate 5-min incubations. Control activity was 0.28 ± 0.01 nmole of epoxide formed in 5 min.

RESULTS

When groups of rats were treated 24 hr before with doses of 4-TCP and Cl-K sufficient to block prothrombin synthesis completely for more than 24 hr [7], the liver epoxidase activities were 40 per cent less than controls in both groups (Table 1). Prothrombin precursor activity was increased in the livers of these groups to a comparable extent as rats treated with warfarin for comparison (Table 1).

Inhibition of phylloquinone epoxidase activity in vitro. To determine the relative effectiveness of 4-TCP and Cl-K in inhibiting epoxidase activity, various concentrations of the anticoagulants were added to epoxidase assay mixtures derived from livers of untreated rats (Fig. 1). As low as 6×10^{-7} M Cl-K in the incubation mixture inhibited epoxidase activity by over 50 per cent and 6×10^{-6} M inhibited by over 80 per cent. Cl-K was about thirty times more potent an inhibitor than 4-TCP; 3×10^{-6} M Cl-K gave approximately the same inhibition (75 per cent) as 10^{-4} M 4-TCP. From Table 2, Cl-K is at least twenty times more effective than 4-TCP in inhibiting prothrombin synthesis over an 8-hr period.

Inhibition of epoxidation and prothrombin synthesis in vivo. In order to test the effect of Cl-K and 4-TCP on epoxidation of vitamin K *in vivo*, reduction of the epoxide was inhibited with warfarin and the conversion of [^{3}H]phylloquinone to [^{3}H]phylloquinone epoxide was measured. Warfarin alone produced an epoxide:K_1 ratio of 4.3 (Table 2). A reduction in this ratio was presumed to have resulted from inhibition of the conversion of [^{3}H]K to [^{3}H]epoxide. Separate experiments were carried out to determine the inhibition of prothrombin synthesis by the anticoagulants to see whether inhibition of epoxidation was correlated with inhibition of prothrombin synthesis. Cl-K at doses of 0.3 and 0.05 and 4-TCP at 6 mg/100 g

Table 1. Effect of anticoagulants on epoxidase and prothrombin precursor activity of rat liver

	Plasma prothrombin (per cent of control)	Epoxidase activity (nmole formed)	Precursor activity (NIH thrombin units‡/mg protein)
Untreated	74 ± 4*	0.27 ± 0.01	<0.2
Cl-K	6 ± 1	0.16 ± 0.01	2.5 ± 0.2
4-TCP	5 ± 1	0.16 ± 0.02	1.8 ± 0.3
Warfarin	17 ± 5	†	1.4 ± 0.2

Rats (6 weeks old) were injected i.p. with 4-TCP (6 mg/100 g body wt), warfarin (0.1 mg/100 g body wt) or i.e. with Cl-K (0.3 mg/100 g body wt). At 24 hr, blood samples were taken for prothrombin assay [12]. The livers were removed and assayed for epoxidase and prothrombin precursor activity (see Materials and Methods). Incubations for epoxidase were for 5 min at 37°. Values are the average ± S.E.M. for 4 rats.

* Control plasma was pooled plasma from twenty 11–12-week old male Sprague Dawley rats. Younger rats as used in these experiments have been observed to have lower plasma prothrombin [19].

† Epoxidase activity was not determined. Data from similar experiments indicate that epoxidase activity in warfarin-treated rats is from 1.5 to 2 times that observed in untreated animals [10].

‡ NIH thrombin units were determined as described in the text by comparison of clotting times of experimental samples to the clotting times of standardized solutions of thrombin.

Table 2. Inhibition of epoxidation of phylloquinone and prothrombin synthesis

Drug administered	Dose (mg/100 g body wt)	Plasma prothrombin* at 8 hr (per cent of control)	Hepatic ratio† [^{3}H]epoxide:[^{3}H]K_1
Control	0	100	4.3 ± 0.6
Cl-K	0.3	46 ± 5	0.71 ± 0.08
	0.05	52 ± 2	1.8 ± 0.5
	0.005	96 ± 9	3.0 ± 0.5
4-TCP	6.0	46 ± 2	1.8 ± 0.4
	1.0	82 ± 6	2.5 ± 0.3
	0.1	106 ± 4	4.3 ± 1.0
Tetrachloro-2-pyridinol	6.0	90 ± 2	2.6 ± 0.2

Rats were killed 2 hr after injection of [^{3}H]K_1 and the livers were analyzed for [^{3}H]K_1 and [^{3}H] epoxide as described previously [1, 5]. The results are the averages ± S.E.M. for 3–7 animals.

* To determine inhibition of prothrombin synthesis, rats were injected i.c. with Cl-K and i.p. with the tetrachloropyridinols and blood samples were taken at 8 hr for prothrombin assay [12]. The control plasma was pooled plasma from twenty 11–12-week old male Sprague–Dawley rats. The values are the averages for 4–8 rats ± S.E.M.

† To determine the inhibition of epoxidation of vitamin K_1, separate groups of rats were injected with warfarin (1 mg/100 g body wt) 5 min after a tracer dose of [^{3}H]K_1 (5 ng/100 g body wt). The other anticoagulant, where indicated, was injected just before the radioactive vitamin. [^{3}H]K_1 and Cl-K were injected i.c. while warfarin and the tetrachloropyridinols were administered i.p.

body wt blocked prothrombin synthesis over 8 hr* and significantly reduced the hepatic epoxide:K_1 ratio ($P < 0.05$). Cl-K was substantially more effective than 4-TCP (Table 2). When Cl-K (0.005 mg/100 g body wt) and 4-TCP (0.1 mg/100 g body wt) were administered at doses which did not lower plasma prothrombin the ratios were not significantly different from the control treated with warfarin alone ($P > 0.1$). 4-TCP at 1 mg/100 g body wt caused a slight inhibition of prothrombin synthesis and the epoxide:K_1 ratio was less than the control ($P < 0.1$). Tetrachloro-2-pyridinol, an isomer of 4-TCP, lowered plasma prothrombin only slightly at the same dose (6 mg/100 g body wt) at which 4-TCP completely blocked prothrombin synthesis. The 2-isomer also decreased the epoxide:K_1 ratio relative to the control ($P < 0.1$). These results show a rough correlation between inhibition of prothrombin synthesis and inhibition of the epoxidation of vitamin K.

Epoxide formation in reversal of inhibition of prothrombin synthesis. Since vitamin K_1, in sufficient amount, can overcome the anticoagulant effects of Cl-K and 4-TCP [14, 15], we compared the amount of vitamin K_1 epoxide formed with the amount of prothrombin synthesized when ineffective and effective doses of vitamin K_1 were administered to anticoagulant-treated rats (Table 3). All rats were treated with warfarin to inhibit reduction of any epoxide formed. Groups given warfarin alone, or warfarin with Cl-K or 4-TCP 24 hr previously were injected with 1 or 200 μg of [^{14}C]phylloquinone. The small dose of phylloquinone did not increase plasma prothrombin and 0.05–0.22 nmole of epoxide were produced in the liver. Two hundred μg of phylloquinone increased plasma prothrombin by 1–3 nmole and 14–31 nmole of epoxide were found in the liver.

*The half-life of prothrombin in the rat, determined by blocking protein synthesis, estimated to be around 7 hr [13].

DISCUSSION

4-TCP, like warfarin and Cl-K [8], caused the accumulation of prothrombin precursor activity in the microsomes, suggesting that 4-TCP also interferes with the conversion of precursor to active plasma prothrombin. However, previous studies indicated that Cl-K and 4-TCP do not have the same mode of action as coumarin and indanedione anticoagulants [7]. Inhibition of prothrombin synthesis was reversed by phylloquinone epoxide in Cl-K and 4-TCP treated rats but not in warfarin and phenylindanedione treated animals because the latter anticoagulants blocked the epoxide-K_1 conversion. Also, rats genetically resistant to warfarin and phenylindanedione were not resistant to 4-TCP and Cl-K [7]. The present results demonstrate that 4-TCP and Cl-K also interfere with the K_1-epoxide cycle but inhibit the epoxidation rather than the reduction step both *in vivo* and *in vitro*. It might be anticipated that Cl-K would inhibit vitamin K metabolism because of structural similarity but the inhibition by 4-TCP, which has no apparent similarity, lends support to the possibility that epoxidation of vitamin K is somehow linked to clotting protein synthesis. In this connection Willingham and Matschiner found that hepatic phylloquinone epoxidase activity was inversely proportional to the concentration of plasma prothrombin and directly proportional to microsomal prothrombin precursor activity [10]. In addition, the *cis* isomer of phylloquinone, which has little or no vitamin K activity [16], is poorly converted to an epoxide either *in vivo* or *in vitro* [17]. It may be significant that the inhibitors of epoxidation, 4-TCP and Cl-K, were more effective than warfarin in inhibiting prothrombin production in an *in vitro* system [18].

If inhibition of epoxidase activity is somehow involved in inhibition of prothrombin synthesis, then the doses of Cl-K and 4-TCP which block prothrombin synthesis must be high enough to inhibit hepatic epoxidase activity. Epoxidase activity assayed *in vitro*

Table 3. Vitamin K_1 and epoxide in liver and prothrombin in plasma 1 hr after administration of vitamin K to anticoagulant-treated rats

Vitamin K administered (nmole)	Anticoagulant administered	Liver		Plasma
		K + Epoxide (nmole)	Epoxide (nmole)	Prothrombin increase* (nmole)
2.2	Cl-K + warfarin	0.39 ± 0.02	0.05 ± 0.01	0
	4-TCP + warfarin	0.38 ± 0.01	0.05 ± 0.01	0
	Warfarin	0.45 ± 0.04	0.22 ± 0.05	0
444	Cl-K + warfarin	123 ± 28	17 ± 4	1.0 ± 0.2
	4-TCP + warfarin	137 ± 18	14 ± 1	2.4 ± 0.1
	Warfarin	182 ± 18	31 ± 4	3.0 ± 0.0

Rats were injected i.p. with 4-TCP (6 mg/100 g body wt), warfarin (0.1 mg/100 g body wt) or i.c. with Cl-K (0.3 mg/100 g body wt). After 24 hr, rats were injected i.v. with either 1 μg (2.2 nmole) or 200 μg (444 nmole) of [^{14}C]phylloquinone. After 1 hr blood samples were taken for prothrombin assay [12] and the livers were removed and analyzed as previously described [1, 5]. Each value represents the average ± S.E.M. for 3 rats.

* Rat prothrombin has a mol. wt of 86,000 [20]; normal rat plasma contains about 200 Iowa units/ml and the sp. act. of purified prothrombin is about 2400 units/mg [21]. Based on these values, plasma (vol = 40 ml/kg) of a 200 g rat contains about 8 nmole prothrombin (1 nmole/ml). From 12–37 per cent of this normal concentration of prothrombin (1–3 nmole) was formed during the experiment.

from rats treated 24 hr previously with Cl-K (0.3 mg/100 g body wt) or 4-TCP (6 mg/100 g body wt) was decreased by 40 per cent. From the data in Fig. 1, 40 per cent inhibition occurs at about 4×10^{-7} M Cl-K and 2×10^{-5} M 4-TCP. The liver was diluted 1:8 in the epoxidase assay so that the concentration in liver after 24 hr would be about 3.2×10^{-6} M Cl-K and 1.6×10^{-4} M 4-TCP which represents 80 per cent inhibition of the epoxidase (Fig. 1). From these calculations, it can also be estimated that less than 1 per cent of the injected anticoagulant was present in the liver after 24 hr. Calculations were made on the assumption that a 200-g rat has a 10-g liver which is 72 per cent water.

When the inhibition of prothrombin synthesis by warfarin, Cl-K or 4-TCP was reversed by vitamin K_1, a substantial amount of phylloquinone epoxide was formed from the vitamin (Table 3). It was calculated that 6–17 nmole of vitamin K epoxide were found in liver for every nmole of prothrombin which appeared in the plasma. The amount of epoxide actually formed during the experiment was probably greater than the amount found in the liver since reduction of the epoxide back to the vitamin is not completely inhibited by warfarin [7]. These results do not provide a reliable estimate of the stoichiometry between vitamin K epoxidation and prothrombin formation but they do show that sufficient epoxide was formed during the synthesis of prothrombin to be consistent with the idea that epoxidation of vitamin K is required for the formation of prothrombin [10]. It may be anticipated that any compounds which interrupt the interconversion of vitamin K and vitamin K epoxide will interfere with the production of prothrombin.

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