INHIBITION OF PROTHROMBIN SYNTHESIS AND EPOXIDATION OF VITAMIN K_1 BY ANTICOAGULANTS IN VITRO

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SUMMARY

A number of 4-hydroxycoumarin and indanedione anticoagulants completly inhibited prothrombin synthesis in vitro at concentrations of less than 10 mM. Since the high concentration of vitamin K_1 used to stimulate prothrombin synthesis made it unlikely that the anticoagulants were inhibiting prothrombin synthesis by preventing the regeneration of vitamin from vitamin K_1 epoxide, this suggested a second site of action of anticoagulants. This second site may be the epoxidation of vitamin K since all the anticoagulants inhibited the epoxidation at concentrations less than 7 mM. 3-Phenyl-4-hydroxycoumarin, Dicumarol, Marcoumar and phenylindanedione were the most potent in inhibiting prothrombin synthesis and epoxidation while Warfarin, 4-hydroxycoumarin and indanedione were the least effective.

Vitamin K_1 undergoes a cyclic conversion to vitamin K_1 epoxide which is reduced back to the vitamin (1-4). The reduction of the epoxide to vitamin K_1 is inhibited by 4-hydroxycoumarin and indanedione anticoagulants and it has been proposed that these anticoagulants block the synthesis of the vitamin K dependent clotting proteins by inhibiting the regeneration of vitamin K (4, 5). The development by Shah and Suttie (6) of a system from rat liver which will synthesize prothrombin when vitamin K_1 is added has provided an excellent tool to further test the hypothesis. If coumarins and indanediones act only by lowering the concentration of vitamin K, then these drugs should not be potent inhibitors in the in vitro system when high concentrations of vitamin K_1 are employed because the regeneration of vitamin from the epoxide would not be important. Indeed, Shah and Suttie found that Warfarin [3-(α-acetonylbenzyl)-4-hydroxycoumarin] at 0.3 mM only slightly inhibited prothrombin synthesis (6). However, we found that a number of 4-hydroxycoumarin and indanedione derivatives at concentrations of 10 mM

or less did inhibit prothrombin synthesis completely in vitro. This suggested that the anticoagulants were inhibiting prothrombin synthesis at a site other than the regeneration of vitamin K from the epoxide. This paper provides evidence that this second site may be the epoxidation of vitamin K.

MATERIALS AND METHODS

Sodium Warfarin was a gift from Endo Laboratories, Coumatetralyl [4-hydroxy-3-(1, 2, 3, 4-tetrahydro-1-napthyl) coumarin] and Marcoumar [3-(α-ethylbenzyl)-4-hydroxycoumarin] from Dr. William Trager, University of Washington, Seattle and 3-phenyl-4-hydroxycoumarin from Dr. James Sadowski, University of Wisconsin, Madison. Dicoumarol [3, 3'-methylenebis (4-hydroxycoumarin)] and 4-hydroxycoumarin were obtained from Sigma Chemical Co. and the indanediones from K & K Laboratories. The indanediones were dissolved in ethanol and the 4-hydroxycoumarins were either dissolved in ethanol or dilute aqueous sodium hydroxide. Vitamin K_1 epoxide was synthesized (7).

6-7- [^{3}H] Vitamin K_1 was a generous gift from Dr. John Matschiner, University of Nebraska School of Medicine, Omaha. It was purified by chromatography on silicic acid (1).

The microsome-cytosol system for measuring prothrombin synthesis and the epoxidation of vitamin K_1 was prepared according to Shah and Suttie (6) except the liver was homogenized with a Polytron homogenizer (Brinkmann Instruments) and centrifuged at 15,000 x g. The rats were fed vitamin K deficient diet (13) for 10-20 days. The incubation mixture was also prepared according to Shah and Suttie (6) except that NADH (1 mg/ml) was added. The mixtures were incubated for 0.5 hr at 27° after the addition of vitamin K_1.

RESULTS

Inhibition of Prothrombin Synthesis in vitro by Coumarins and Indanediones

The in vitro production of prothrombin was stimulated by an energy source and by reduced pyridine nucleotides as previously reported (6, 8). Warfarin was a weak inhibitor of prothrombin synthesis but was effective at concentrations above 10 mM (Fig 1). All the other anticoagulants were inhibitory at lower concentrations. 3-Phenyl-4-hydroxycoumarin, the most potent inhibitor, reduced prothrombin synthesis by about 40% at a concentration less than 0.1 mM.

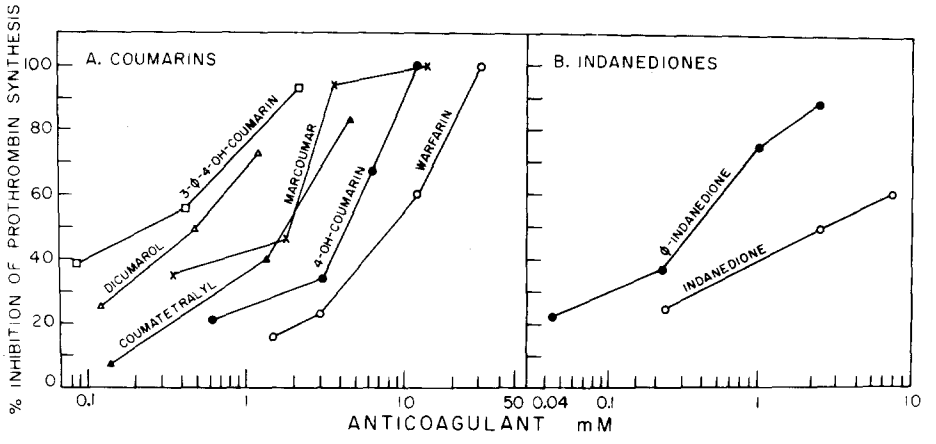

Fig 1A and B Inhibition of prothrombin synthesis in vitro by 4-hydroxycoumarins and indanediones. Vitamin K_1 (Nutritional Biochemicals) in ethanol was added to a final concentration of 20 μg/ml in the incubation mixture as described in Materials and Methods. After shaking for 0.5 hr at 27° the flasks were cooled in an ice bath and the microsomal fraction prepared according to Shah and Suttie (6). Prothrombin activity was assayed by the two-stage method of Shapiro and Waugh (14). Clotting times of dilutions of a solution of NIH standard thrombin in 0.9% NaCl were used to calculate prothrombin concentrations in NIH units of thrombin activity. In the control flasks without anticoagulant 5.1 to 7.5 units of prothrombin were synthesized per gram of liver. The % inhibitions shown are the average for at least 3 different liver preparations. The 4-hydroxycoumarins tested are shown in Fig 1A: Warfarin o 4-hydroxycoumarin ● Coumatetralyl ▲ Marcoumar X Dicoumarol △ 3-phenyl-4-hydroxycoumarin □ . The indanediones tested are shown in Fig 1B: 2-phenyl-1,3-indanedione ● and 1,3 indanedione o.

Warfarin and the Regeneration of Vitamin K_1 from Vitamin K_1 Epoxide

The possibility that the anticoagulants were blocking prothrombin synthesis by inhibiting the regeneration of vitamin K_1 from vitamin K_1 epoxide was made unlikely by the observation that Warfarin at 6 μM inhibited by 86% the production of prothrombin stimulated by epoxide (200 μg/ml). Since it required a concentration of Warfarin about 500 times greater to inhibit prothrombin synthesis in vitro (Fig 1) it is unlikely that the inhibition of the epoxide to K_1 conversion is important in the inhibition of prothrombin synthesis in the presence of high levels of vitamin K_1 (20 μg/ml).

Inhibition of Epoxidation of Vitamin K_1 by Coumarins and Indanediones

Since it was proposed that the epoxidation of vitamin K was somehow

linked to the conversion of prothrombin precursor to prothrombin (4, 9), we tested the effects of anticoagulants on the conversion of vitamin K_1 to vitamin K_1 epoxide in the in vitro system. All the 4-hydroxycoumarins and indanediones inhibited the epoxidation of vitamin K_1 significantly at concentrations less than 7 mM (Table I). 3-Phenyl-4-hydroxycoumarin, among the

Table I Inhibition of Epoxidation of Vitamin K_1 by Anticoagulants

Anticoagulant	Concentration mM	% Inhibition of Epoxide Formation	% Inhibition of Prothrombin Synthesis[1]
Coumarins			
Warfarin	30	59 ± 9	100
	3.0	32 ± 4	23
3-Phenyl-4-	2.1	67 ± 3	93
hydroxycoumarin	0.42	39 ± 5	55
Marcoumar	3.5	84 ± 7	94
	0.35	39 ± 5	35
Coumatetralyl	3.4	51 ± 8	83
4-Hydroxycoumarin	6.2	18 ± 2	67
	0.62	4 ± 4	21
Dicumarol	1.2	55 ± 9	73
	0.12	18 ± 5	24
Indanediones			
Indanedione	6.8	30 ± 9	61
	2.3	20 ± 10	50
Phenylindanedione	2.2	77 ± 4	89
	0.22	24 ± 9	37

[1]These values are taken from Fig 1.

Three μg of [^{3}H] K_1 (10^6 dpm) in 0.05 ml of ethanol were added to 5 ml of incubation mixture (see Materials and Methods). After shaking for 0.5 hr at 27° the reaction was terminated by the addition of 7 ml of isopropanol:hexane (3:2). The amount of conversion of [^{3}H] K_1 to ^{3}H epoxide was determined as previously described (9). The activity of the control incubation mixture varied between 0.6 and 1.3 nmoles of epoxide produced per gram of liver. The % inhibition of epoxidation by the anticoagulants was the average of at least 3 determinations done in duplicate on three different liver preparations.

most effective inhibitors, inhibited epoxidation by 39% at 0.4 mM and 67% at 2.1 mM. In contrast, Warfarin at 3 mM caused a 32% inhibition and 30 mM was required for a 59% inhibition of epoxidation.

DISCUSSION

We were surprised to find that coumarin and indanedione anticoagulants inhibited the synthesis of prothrombin *in vitro* since the amount of vitamin K_1 added was high (20 μg/ml). In experiments *in vivo* we found that the stimulation of prothrombin synthesis by a dose of vitamin K_1 of 50 μg/100 gbw[1] was not inhibited by as much as 1 mg/100 gbw of warfarin (2). This is presumably because at high concentrations of vitamin K in the liver, the inhibition of the regeneration of vitamin from vitamin K epoxide does not lower the concentration to an ineffectual level as it would if the initial concentration of vitamin were low.

Also unexpected was the observation that 3-phenyl-4-hydroxycoumarin was the most potent inhibitor of prothrombin synthesis *in vitro* since it was one of the weakest inhibitors *in vivo* among the anticoagulants tested (10). Warfarin was one of the most potent inhibitors *in vivo* (10) and was the weakest *in vitro* (Fig 1). Sadowski and Suttie (11) and Bell et al. (10) found that 3-phenyl-4-hydroxycoumarin was a poor inhibitor of the conversion of epoxide to vitamin K_1 *in vivo* and *in vitro* while Warfarin was one of the most potent inhibitors of this reduction. These findings appear to be reconciled by the observation that 3-phenyl-4-hydroxycoumarin is a potent inhibitor of the epoxidation of vitamin K while Warfarin is much less inhibitory (Table I).

There was fairly good agreement in the order of potency of the anticoagulants in inhibiting prothrombin synthesis and epoxidation *in vitro*. 3-Phenyl-4-hydroxycoumarin, Dicoumarol, Marcoumar and phenylindanedione were the most potent in inhibiting prothrombin synthesis and epoxidation while Warfarin, 4-hydroxycoumarin and indanedione were the least potent. There was also a rough correlation between inhibition of epoxidation and inhibition of prothrombin synthesis. Concentrations of the anticoagulants which inhibited epoxidation always inhibited prothrombin synthesis.

These experiments raise the possibility that anticoagulants may inhibit

[1] grams body weight

both the epoxidation and regeneration of vitamin K in vivo and that both these processes are necessary for normal clotting protein synthesis. The dose of Warfarin required to block prothrombin synthesis completely over 8 hr is about 35 μg/100 gbw (12). If 10% of the injected drug was in the liver (3) the Warfarin concentration would be approximately 0.04 mM which corresponds to less than a 30% inhibition of epoxidation (Table I) and prothrombin synthesis (Fig 1). Therefore Warfarin probably does not inhibit epoxidation of vitamin K in vivo at doses which block prothrombin synthesis. On the other hand, a dose of 6 mg/100 gbw of 3-phenyl-4-hydroxycoumarin is required for complete inhibition of prothrombin synthesis over 8 hr (10). If 10% of this drug was in the liver the concentration would be approximately 10 mM. In the in vitro system a concentration of 2.1 mM inhibited epoxidation by 67% and prothrombin synthesis by 93%. A 1 mg/100 gbw dose of Dicoumarol is required for complete inhibition of prothrombin synthesis and this would correspond to a liver concentration of 1.1 mM. In the in vitro system a concentration of 1.2 mM inhibited prothrombin synthesis by 73% and epoxidation by 55%. Hence, 3-phenyl-4-hydroxycoumarin and Dicoumarol may block prothrombin synthesis in vivo indirectly by inhibiting the epoxidation of vitamin K in addition to inhibiting the regeneration of vitamin K from its epoxide.

This paper adds further support to the idea that the vitamin K - K epoxide cycle is involved in prothrombin synthesis. The evidence points to the epoxidation of vitamin K as the step linked to prothrombin synthesis with the reduction step providing for the regeneration of vitamin K.

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