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COUMARINS AND THE VITAMIN K–K EPOXIDE CYCLE. LACK OF RESISTANCE TO COUMATETRALYL IN WARFARIN-RESISTANT RATS

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Abstract—In female warfarin-resistant rats, coumatetralyl at 0.5 mg/100 g body wt blocked prothrombin synthesis and interrupted the vitamin K_1–K_1 epoxide cycle by almost completely blocking the conversion of epoxide to vitamin K_1. In contrast, prothrombin synthesis and the epoxide–K_1 conversion were unaffected by warfarin at the same dose, although at 2 mg/100 g body wt warfarin also blocked prothrombin synthesis and the conversion. In Sprague–Dawley rats, the anticoagulants inhibited prothrombin synthesis about equally well over a range of doses. At 0.05 mg/100 g body wt or greater, warfarin and coumatetralyl severely inhibited both prothrombin synthesis and the reduction of the epoxide to K_1, while at 0.01 mg/100 g body wt the anticoagulants had little effect.

Metabolic studies with tracer doses of [^{3}H]K_1 and [^{3}H]epoxide indicated that resistant rats have hepatic epoxide: K_1 ratios 5–6-fold greater than in Sprague Dawley animals. The hepatic concentrations of [^{3}H]K_1 in male and female resistant rats were 41 and 26 per cent, and plasma prothrombin concentrations 17 and 39 per cent respectively, of those values in Sprague–Dawley rats. When resistant rats were injected with vitamin K_1, plasma prothrombin increased while the hepatic epoxide: K_1 ratio decreased. Two days later prothrombin and the ratio had returned to their original values.

These results are consistent with the idea that the K_1 epoxide cycle is involved in clotting protein synthesis and that the site of action of coumarins is the epoxide–K_1 conversion. The impaired epoxide–K_1 conversion may explain why warfarin-resistant rats have a lowered rate of prothrombin synthesis.

It was proposed that coumarin and indanedione anticoagulants inhibit clotting protein synthesis by causing the accumulation of vitamin K_1 epoxide, a metabolite and inhibitor of the vitamin [1–3]. Recently it was observed that there is a lack of correlation between the relative amounts of epoxide* and vitamin K_1 in the liver and inhibition of prothrombin synthesis [4–6]. However, the interconversion of vitamin K_1 and epoxide is very likely involved in the mechanism of warfarin (3-(α-acetonylbenzyl)-4-hydroxycoumarin) action since in warfarin-resistant rats the inhibition of the epoxide–K_1 conversion by warfarin was greatly reduced [7, 8]. Another 4-hydroxycoumarin, coumatetralyl (4-hydroxy-3-(1,2,3,4-tetrahydro-1-napthyl)coumarin) was reported to be substantially more toxic than warfarin to warfarin-resistant rats [9]. Martin subsequently found that coumatetralyl was about six times more effective than warfarin in blocking clotting protein synthesis in the resistant strain but was about twice as effective in normal laboratory rats [10]. If the locus of action of coumarins is the epoxide–K_1 conversion, then coumatetralyl should be much more effective than warfarin in blocking this conversion in resistant rats and slightly more effective in Sprague–Dawley animals. We investigated the K_1–epoxide cycle by injecting tracer doses of [^{3}H]K_1 and [^{3}H]epoxide into male and female resistant rats in order to ascertain the relative endogenous amounts of vitamin K_1 and epoxide and to determine if there is a correlation with prothrombin synthesis. We then compared the effect of warfarin and coumatetralyl on the cycle and prothrombin synthesis in Sprague–Dawley and resistant animals.

*Epoxide stands for vitamin K_1 epoxide and K_1 for vitamin K_1 in this paper.

MATERIALS AND METHODS

6,7-[^{3}H]Vitamin K_1 was obtained and purified as described [11, 12]. Tritiated vitamin K_1 epoxide was prepared according to Tishler *et al.* [13]. Tween emulsions of [^{3}H]K_1 and [^{3}H]epoxide (5 ng/100 g body wt) were injected as tracer doses. Coumatetralyl and sodium warfarin were generous gifts from Mr. Lance Pohl and Dr. William Trager of the University of Washington, Seattle and Endo Laboratories (Garden City, N.Y.), respectively. Coumatetralyl was dissolved in dilute NaOH solution and the pH adjusted to 7.5 wtih HCl. Plasma prothrombin was assayed by the method of Hjort *et al.* [14] and control plasma was pooled plasma from twenty 11 12-week old male Sprague–Dawley rats. The results are expressed as per cent of control prothrombin although the assay is somewhat sensitive to the concentration of Factor X which is also a vitamin K-dependent factor.

Warfarin-resistant (10–15-week old) rats, obtained as described previously [7], and Sprague–Dawley rats (10–15-weeks old) from Charles River Laboratories were used in these experiments. They were fed Purina rat chow unless it is stated otherwise.

RESULTS

Male warfarin-resistant rats. The hepatic epoxide: K_1 ratio, was 6-fold greater in resistant rats than was found previously in Sprague–Dawley animals [12] (Table 1). The [^{3}H] K_1 concentration in resistant rats

Table 1. Metabolism of tracer doses of [^{3}H]K_1 in resistant rats

	Warfarin (mg/100 g body wt)	Per cent injected ^{3}H in [^{3}H]K_1	Epoxide: K_1 ratio	Prothrombin per cent of control‡
Male, resistant				
Fed Purina chow		3.5 ± 0.7	0.94 ± 0.11	17 ± 3
	0.1	5.3 ± 0.5	1.6 ± 0.1	
	5.0	3.5 ± 0.2	3.5 ± 0.2	
Female, resistant				
Fed Purina chow		3.4 ± 0.6	0.96 ± 0.12	62 ± 4
Fed Purina chow and injected with vitamin K*		8.1 ± 0.2	0.34 ± 0.03	132 ± 10
Fed vitamin K-deficient diet†		3.3 ± 0.6	0.93 ± 0.13	16 ± 6
Female, Sprague–Dawley				
Fed Purina chow		13.2 ± 3.0	0.18 ± 0.04	159 ± 8

Rats were injected i.c. with tracer doses of [^{3}H]K_1 and killed 2 hr later. Where indicated warfarin was injected i.p. 0.5 hr before the labeled vitamin or epoxide. Livers were analyzed as described previously [7]. The results are the mean ± S.E.M. for 3–8 rats.

* Rats were injected intramuscularly with vitamin K_1 (40 μg/100 g body wt) 16 hr before injection with a tracer dose of [^{3}H]K_1 to determine the epoxide: K_1 ratio.

† Rats were fed vitamin K-deficient diet (prepared by General Biochemicals, Chagrin Falls, Ohio according to Matschiner and Taggart [23]) for 4 days.

‡ Control plasma was pooled plasma from male rats.

was 41 per cent of that found in Sprague–Dawley rats. A dose of warfarin (0.1 mg/100 g body wt), which blocked prothrombin synthesis and increased the epoxide: K_1 ratio almost 20-fold in Sprague–Dawley rats [12], did not block prothrombin synthesis [12] and increased the ratio by less than 2-fold in resistant animals (Table 1). Warfarin at 5 mg/100 g body wt blocked prothrombin synthesis in resistant rats [7] and increased the ratio to 3.5 (Table 1), which is similar to that found in Sprague–Dawley rats treated with 0.1 mg/100 g body wt of warfarin [12]. Experiments with tracer doses of [^{3}H]epoxide confirmed these results. After administration of either labeled K_1 or epoxide to Sprague–Dawley and resistant rats the K_1 epoxide cycle comes to equilibrium at epoxide: K_1 ratios of about 0.2 and 1, respectively. After warfarin (0.1 mg/100 g body wt) treatment the ratios were approximately 3.0 and 1.6 respectively.

When male resistant rats were injected with a near-minimum effective dose of [^{3}H]K_1 (3 μg/100 g body wt), plasma prothrombin increased rapidly and the hepatic epoxide: K_1 ratio decreased to 0.37 at 2 hr (Fig. 1). The ratio was 0.16 in Sprague–Dawley males which have normal prothrombin levels [12]. In resistant animals the ratio was 0.48 at 11 hr after which plasma prothrombin declined. At 48 hr the prothrombin concentration and the ratio had returned to approximately their zero-time values.

Table 2. Inhibition of prothrombin synthesis and the vitamin K_1 epoxide–vitamin K_1 conversion in female Sprague–Dawley and resistant rats

	mg/100 g body wt	Per cent control prothrombin at 8 hr	Hepatic ratio epoxide: K_1
Sprague–Dawley			
Control	0	159 ± 8	0.30 ± 0.06
Warfarin	0.5	81 ± 7	6.3 ± 0.9
	0.05	93 ± 4	2.1 ± 0.4
	0.01	141 ± 8	0.43 ± 0.12
Coumatetralyl	0.5	82 ± 2	20 ± 1
	0.05	83 ± 5	4.5 ± 0.4
	0.01	146 ± 7	0.41 ± 0.02
Resistant			
Control	0	146 ± 11	1.9 ± 0.2
Warfarin	2.0	69 ± 10	5.1 ± 0.8
	0.5	150 ± 10	1.8 ± 0.2
Coumatetralyl	2.0	72 ± 2	
	0.5	73 ± 5	17 ± 4
	0.05	104 ± 11	

To determine inhibition of prothrombin synthesis, rats were injected i.p. with the anticoagulants at zero-time. The resistant rats were injected intramuscularly with vitamin K_1 in a Tween emulsion (25 μg/100 g body wt) 16 hr before zero-time in order to increase plasma prothrombin to levels found in Sprague–Dawley animals. The values are the mean ± S.E.M. for 4–8 rats.

For determination of the inhibition of the epoxide–K_1 conversion, rats were injected i.c. with a tracer dose of [^{3}H]epoxide and killed 2 hr later. Where indicated warfarin or coumatetralyl were injected i.p. 2 hr before the labelled epoxide. Livers were analyzed as described previously [7]. The results are the mean ± S.E.M. for 3 rats.

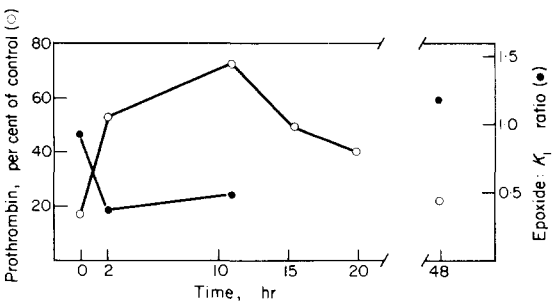

Fig. 1. Hepatic epoxide: K_1 ratios and plasma prothrombin in male resistant rats. Groups of rats were injected i.v. with [^{3}H]K_1 (3 μg/100 g body wt) and killed at times indicated for liver analysis to determine the epoxide: K_1 ratios (●) [7]. Blood samples were taken at times indicated for prothrombin assays (○). The values are the averages for 3–4 animals.

Female warfarin-resistant rats. Resistant females also had elevated levels of epoxide relative to vitamin K_1 and prothrombin concentrations 39 per cent of those found in Sprague–Dawley females. The hepatic epoxide: K_1 ratio was about 5-fold higher and the [^{3}H]K_1 concentration was 26 per cent of that found in Sprague–Dawley rats (Table 1). These results were confirmed by studies with tracer doses of [^{3}H]epoxide. Resistant females were injected with vitamin K_1 (40 μg/100 g body wt) which increased plasma prothrombin and decreased the hepatic epoxide: K_1 ratio over 16 hr (Table 1). When female rats were fed a vitamin K-deficient diet, plasma prothrombin decreased but the hepatic epoxide: K_1 ratio did not change significantly.

Effect of warfarin and coumatetralyl on prothrombin synthesis and epoxide–K_1 conversion. In Sprague–Dawley rats, warfarin and coumatetralyl blocked prothrombin synthesis effectively over 8 hr at 0.5 mg and 0.05 mg/100 g body wt (Table 2). With these doses the half-life of plasma prothrombin was calculated to be 7.7 to 10.3 hr. The half-life of prothrombin in the rat, determined by blocking protein synthesis, was estimated to be 5.3–7.0 hr [15]. The epoxide–K_1 conversion was also greatly inhibited over 4 hr by both anticoagulants (Table 2). Coumatetralyl was more effective than warfarin. At 0.01 mg/100 g body wt both anticoagulants had little effect on either prothrombin synthesis or the epoxide–K_1 conversion.

In warfarin-resistant rats, warfarin had no effect on prothrombin synthesis or the epoxide–K_1 conversion at 0.5 mg/100 g body wt but at 2 mg/100 g body wt both prothrombin synthesis and the conversion were clearly inhibited. Coumatetralyl at 0.5 mg/100 g body wt blocked both prothrombin synthesis and the conversion.

DISCUSSION

The correlation of inhibition of prothrombin synthesis with the blocking of the epoxide–K_1 conversion by coumatetralyl in warfarin-resistant rats provides additional evidence that anticoagulant resistance is due to loss of sensitivity of this reduction to certain coumarins and indanediones [7, 8, 12]. In Sprague–Dawley animals, coumatetralyl and warfarin blocked prothrombin synthesis about equally well over a range of doses. At 0.05 and 0.5 mg/100 g body wt both anticoagulants blocked prothrombin synthesis and clearly inhibited the epoxide–K_1 conversion while at 0.01 mg/100 g body wt they had little effect. In contrast, coumatetralyl at 0.5 mg/100 g body wt blocked prothrombin synthesis and the epoxide–K_1 conversion in warfarin-resistant rats while the same dose of warfarin had no effect on the synthesis or conversion. In agreement, Martin [10] found that coumatetralyl was about as effective as warfarin in blocking prothrombin synthesis in normal laboratory rats but was much more effective in resistant animals. However, he also found that coumatetralyl was twenty times more effective in laboratory rats than in resistant animals whereas our studies indicate that it was only slightly more effective. The site of action for coumarin anticoagulants appears to be altered in resistant rats so that the relative affinities of coumarins have been changed.

Sadowski and Suttie [6] compared the effects of warfarin and three other coumarins on the K_1–epoxide cycle and prothrombin synthesis in laboratory rats. They found that the least effective coumarin in blocking prothrombin synthesis was also the least effective in inhibiting the reduction of vitamin K_1 epoxide to vitamin K_1.

Previous results with large doses of [^{3}H]K_1 and [^{3}H]epoxide indicated that the conversion of epoxide to K_1 was much less sensitive to warfarin in warfarin-resistant rats [7]. Studies with tracer doses of the labeled compounds confirmed this and in addition showed clearly that the K_1–epoxide cycle was altered in resistant animals in the presence or absence of warfarin. In resistant rats, the epoxide: K_1 ratio was elevated as though they had been treated with warfarin. This may explain the observation of Shah and Suttie [17] that resistant rats respond to vitamin K and the 2-chloro-analog of the vitamin as though they had been treated with warfarin. When the low prothrombin levels of resistant rats were increased by vitamin K_1 administration, the epoxide: K_1 ratios decreased. Presumably, vitamin K_1 epoxidase and other liver enzymes that metabolize vitamin K were saturated by the administered vitamin and the epoxide: K_1 ratio decreased. If the K_1–epoxide cycle enzymes were not saturated in the resistant animals fed Purina chow, then it is not surprising that feeding a vitamin K-deficient diet did not increase the epoxide: K_1 ratio still further (Table 1).

In male and female resistant rats the hepatic concentrations of [^{3}H]K_1 were 41 and 26 per cent, respectively, of those values found in Sprague–Dawley animals [12] (Table 1). This decrease, in the most part, can be accounted for by the change in the equilibrium of the K_1–epoxide cycle. The reduced vitamin level may be the cause of or at least contribute to the reduced rate of prothrombin synthesis in resistant rats. Although Hermodson *et al.* [16] found that male resistant rats required the administration of 20 fold more vitamin K_1 than normal laboratory rats to maintain normal prothrombin levels, it is not known how much the actual vitamin K level in the liver was increased. If the cycle is impaired either by warfarin or by a less efficient reductase, more vitamin K is required for normal prothrombin synthesis. Even if the epoxide–K_1 conversion is severely inhibited by

warfarin, prothrombin synthesis still occurs if sufficient vitamin is supplied. Thus, epoxidation of vitamin K may be necessary for prothrombin synthesis, as suggested by the observations that (1) inhibitors of epoxidation are also anticoagulants [18], (2) there is an inverse relationship between epoxidase activity and plasma prothrombin [19], (3) *cis*-vitamin K_1 has little vitamin K activity [20] and is a poor substrate for epoxidation [21] and (4) epoxidation and prothrombin synthesis are tightly coupled *in vitro* [22].

If epoxidation of vitamin K is necessary for prothrombin synthesis, then coumarins probably act by cutting down the supply of vitamin from the epoxide. Warfarin resistance results from loss of sensitivity of the epoxide–K_1 conversion to warfarin and a relative lack of coumatetralyl resistance results from inhibition by this anticoagulant of reduction of the epoxide.

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REFERENCES

1. R. G. Bell and J. T. Matschiner, *Archs Biochem. Biophys.* **141,** (1970) 473.
2. R. G. Bell and J. T. Matschiner, *Nature, Lond.* **237,** (1972) 32.
3. R. G. Bell, J. A. Sadowski and J. T. Matschiner, *Biochemistry* **11,** (1972) 1959.
4. P. T. Caldwell, P. Ren and R. G. Bell, *Biochem. Pharmac.* **23,** (1974) 3353.
5. S. R. Goodman, R. M. Houser and R. E. Olson, *Biochem. biophys. Res. Commun.* **61,** (1974) 250.
6. J. A. Sadowski and J. W. Suttie, *Biochemistry* **13,** (1974) 3696.
7. R. G. Bell and P. T. Caldwell, *Biochemistry* **12,** (1973) 1759.
8. A. Zimmerman and J. T. Matschiner, *Biochem. Pharmac.* **23,** (1974) 1033.
9. J. H. Greaves and P. Ayres, *J. Hyg., Camb.* **67** (1969) 311.
10. A. D. Martin, *Biochem. Soc. Transactions, Lond.* **1,** (1973) 1206.
11. J. T. Matschiner, R. G. Bell, J. M. Amelotti and T. E. Knauer, *Biochim. biophys. Acta* **201,** (1970) 309.
12. P. Ren, R. E. Laliberte and R. G. Bell, *Molec. Pharmac.* **10,** (1974) 373.
13. M. Tishler, L. E. Fieser and N. L. Wendler, *J. Am. chem. Soc.* **62,** (1940) 2866.
14. P. Hjort, S. I. Rapaport and P. A. Owren, *J. Lab. clin. Med.* **46,** (1955) 89.
15. R. G. Bell and J. T. Matschiner, *Archs Biochem. Biophys.* **135,** (1969) 152.
16. M. A. Hermodson, J. W. Suttie and K. P. Link, *Am. J. Physiol.* **217,** (1969) 1316.
17. D. V. Shah and J. W. Suttie, *Proc. Soc. exp. Biol. Med.* **143,** (1973) 775.
18. A. K. Willingham, R. E. Laliberte, R. G. Bell and J. T. Matschiner, *Fedn Proc.* **33,** (1974) 672.
19. A. K. Willingham and J. T. Matschiner, *Biochem. J., Lond.* **140,** (1974) 435.
20. J. T. Matschiner and R. G. Bell, *J. Nutr.* **102,** (1972) 625.
21. T. E. Knauer, A. K. Willingham and J. T. Matschiner, *Fedn Proc.* **33,** (1974) 314.
22. J. A. Sadowski, *Fedn Proc.* **34,** (1975) 898.
23. J. T. Matschiner and W. V. Taggart, *J. Nutr.* **94,** (1968) 57.