J. Pharm. Pharmacol. 1980, 32: 828–832 Received May 6, 1980

Age-dependent differences in the effect of phenprocoumon on the vitamin K_1-epoxide cycle in rats†

DIETMAR TRENK, DIETER BEERMANN, FRANZ OESCH AND EBERHARD JÄHNCHEN*

Pharmakologisches Institut der Universität Mainz, Obere Zahlbacher Straße 67, D-6500 Mainz, Germany

The anticoagulant activity and the pharmacokinetics of phenprocoumon as well as the effect of phenprocoumon on the vitamin K_1-epoxide cycle in younger (12 weeks) and older (36 weeks) male inbred Lewis rats has been examined in a study of the mechanism responsible for the increase in the responsiveness to oral anticoagulant drugs (OAD's) with increasing age. After a single i.v.-dose of phenprocoumon (0·355 mg kg^{-1}) the anticoagulant effect obtained was greater in older than in younger rats. There were no differences between younger and older rats in the rate of elimination, volume of distribution and in the free fraction and free concentration values of phenprocoumon in plasma and liver. After i.v.-injection of 64·3 $\mu g\ kg^{-1}$ [^{3}H]vitamin K_1 and different doses of phenprocoumon (0·02 to 3 mg kg^{-1}) the [^{3}H]vitamin K_1 concentration in the liver decreased and the [^{3}H] vitamin K_1-2, 3-epoxide concentration increased dependent on the dose and the liver concentration of phenprocoumon. These changes were more pronounced in the older than in the younger rats. Concentration-response curves gave similar EC50-values for both age-groups but a 1·6-fold higher maximal response (expressed as vitamin K_1-epoxide/vitamin K_1 ratios) in the older rats. Since OAD's exert their anticoagulant effect most probably by inhibiting an enzyme (vitamin K_1-epoxide reductase) which regenerates vitamin K_1 from the epoxide metabolite and since the vitamin K_1-epoxide/vitamin K_1 ratios in the liver may reflect the degree of inhibition of the epoxide reductase by OAD's, our results may indicate that the inhibitory effect of phenprocoumon on this enzyme is more pronounced in older than in younger rats. This could explain the age-dependent differences in the anticoagulant activity.

Patients and rats in old age are more sensitive to oral anticoagulant drugs than those of younger age (Shepherd et al 1977; Husted & Andreasen 1977; Routledge et al 1979). It was suggested that this increase in responsiveness with age is due to differences in the sensitivity of the receptor site for anticoagulant drugs, but conclusive studies are lacking.

Since the hypoprothrombinaemic effect of coumarin drugs is closely linked to the metabolism of vitamin K_1 (Bell & Matschiner 1972; Willingham & Matschiner 1974; Ren et al 1974; Whitlon et al 1978) we have studied the effect of phenprocoumon on the metabolism of vitamin K_1 in rats of different ages.

METHODS

Materials

(±)-[^{3}H]Phenprocoumon (specific activity 773 $\mu Ci\ mg^{-1}$) and [^{3}H]vitamin K_1 (specific activity 730 $\mu Ci\ mg^{-1}$) were gifts from Hoffmann-La Roche Ltd., Basel, Switzerland. Vitamin K_1-2,3-epoxide was synthesized from vitamin K_1 by oxidation with alkaline H_2O_2 solution in ethanol (Tishler et al 1940).

Male inbred Lewis rats (Zentralinstitut für Versuchstiere, Hannover, Germany) aged 12 and 36 weeks were used. The animals had free access to food (Altromin, Lage, Germany) and water.

Drug assay

[^{3}H]Phenprocoumon was determined by liquid scintillation counting in a Packard Tri-Carb Spectrometer 3375 following specific extraction of plasma and liver samples of 0·1 ml with n-heptane (Schmidt & Jähnchen 1979). Unlabelled phenprocoumon in the livers of the rats which received [^{3}H]vitamin K_1 and phenprocoumon was determined by a fluorimetric assay described earlier (Schmidt & Jähnchen 1977). Vitamin K_1 and its metabolites do not interfere with this assay.

[^{3}H]Vitamin K_1 and [^{3}H]vitamin K_1-2,3-epoxide was determined in samples of 0·1 ml liver homogenate. The samples were diluted with 0·5 ml of distilled water and extracted with 2·0 ml n-hexane–

† Supported by the Deutsche Forschungsgemeinschaft, part of the Ph.D. Thesis of D.T.

* Correspondence.

propanol (2) 3:1 (v/v). After centrifugation 1·0 ml of the hexane phase was removed and evaporated under nitrogen. [^{3}H]Vitamin K_1 and its 2,3-epoxide metabolite were separated by reversed phase thin-layer chromatography (Shearer et al 1973) and determined by liquid scintillation counting. The concentration of [^{3}H]vitamin K_1 and [^{3}H]vitamin K_1-epoxide was expressed as d min^{-1} g^{-1} liver. Total recovery of vitamin K_1 from liver homogenate was 61·1 ± 1·9%. Total radioactivity in the liver was determined by adding 100 μl of liver homogenate to 10 ml of scintillation fluid. Thereafter the samples were rotated for 30 min on a rotary extractor and counted. The recovery by this method was complete.

Plasma protein binding of phenprocoumon was determined by equilibrium dialysis in the samples obtained from the aortic blood. The plasma was dialysed for 16 h at 37 °C against phosphate buffer (0·15 M; pH 7·4); cellophane dialysis membrane (Union Carbide Corp., Chicago, Ill.) was used.

The prothrombin complex activity (PCA) was measured in plasma samples of 0·01 ml as described by Schmidt & Jähnchen (1977).

Pharmacokinetic analysis

Pharmacokinetic parameters of phenprocoumon were estimated according to a one-compartment model. The free fraction of phenprocoumon in the liver tissue (f_L) was calculated assuming that the free concentration in tissue water is equal to the free concentration in the plasma water (Levy et al 1978): $f_L = 0{\cdot}7 \times f_p \times C_p/C_L$, where f_p is the free fraction of phenprocoumon in the plasma, C_p/C_L the plasma/liver concentration ratio and 0·7 the average fraction of water content of the liver. The area of the prothrombin complex activity (PCA) vs time curve (compare Fig. 1) was taken as a measure of the total anticoagulant effect of phenprocoumon per dose. For this purpose the PCA-values obtained after injection of phenprocoumon were subtracted from the predrug value (PCA^0) and the area was estimated by the trapezoid rule.

Animal experiments

In the first study, 6 younger and 6 older rats received a single injection of phenprocoumon (0·355 mg kg^{-1} containing 70·5 μCi kg^{-1} [^{3}H]phenprocoumon) into the penile vein. Thereafter, blood samples were drawn frequently up to 54 h by puncture of the tail artery under slight ether anaesthesia. The animals were killed 54 h after the injection by withdrawal of all blood from the abdominal aorta. The livers were removed, blotted under slight pressure and homogenized with an Ultra-Turrax (Janke & Kunkel KG, Staufen i.Br., Germany) in ice-cold sodium chloride solution (1:4 w/w). This study was designed to investigate the pharmacokinetics and the anticoagulant effect of phenprocoumon in younger and older rats.

In the second study 10 animals of younger age and 11 animals of older age received a single injection of 64·3 μg kg^{-1} (47 μCi kg^{-1}) [^{3}H]vitamin K_1 into the penile vein. In addition, these rats were treated with different doses of unlabelled phenprocoumon (dissolved in 0·9% NaCl (saline) 30 min before the vitamin K_1 dose by injection into the penile vein. The doses of phenprocoumon consisted of 0·02, 0·1, 0·2, 0·3, 0·5, 0·8, 2·0 and 3·0 mg kg^{-1}. Control rats received saline. The rats were killed 6 h after injection of vitamin K_1 by withdrawal of all blood from the abdominal aorta. The livers were removed and homogenized as described above.

RESULTS

The mean time course of the anticoagulant effect obtained in 6 younger and 6 older rats after a single i.v.-dose (0·355 mg kg^{-1}) of phenprocoumon is shown in Fig. 1. It is evident that the depression of prothrombin complex activity is more pronounced in the older than in the younger rats resulting in a significantly greater area above the anticoagulant effect vs time curve for the older rats. These areas were 3419 (442) (% of normal) h^{-1} and 2529 (361) (% of normal) h^{-1} (mean with s.d.) for the older and younger rats, respectively ($P < 0{\cdot}005$ by unpaired Student's t-test).

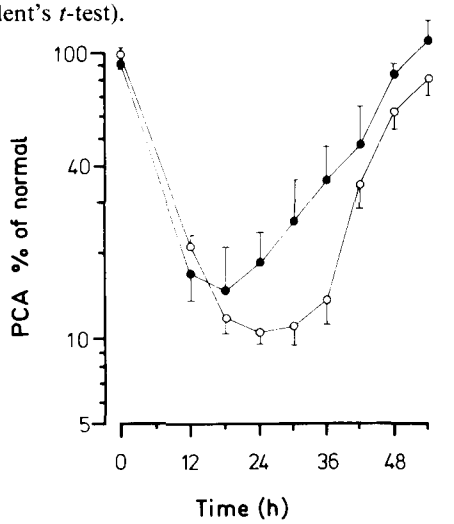

FIG. 1. Time course of prothrombin complex activity (PCA) in the plasma of 6 younger (●) and 6 older (○) male inbred Lewis rats following a single i.v. injection phenprocoumon (0·355 mg kg^{-1}). Mean ± s.e.

Table 1. Pharmacokinetic parameters of phenprocoumon in younger and older male rats (n = 6, mean with s.d.).

Parameters	Younger rats	Older rats	*t*-test
Age, weeks	12	36	—
Body weight, g	295 s.d. 7	431 s.d. 7	$P < 0.001$
Liver weight, % of body weight	2·96 s.d. 0·10	2·35 s.d. 0·12	$P < 0.001$
Half-life, h	17·5 s.d. 1·8	17·6 s.d. 1·3	NS
Free fraction in plasma[a] × 100	1·24 s.d. 0·07	1·27 s.d. 0·11	NS
Volume of distribution, ml kg^{-1}	619 s.d. 105	616 s.d. 66	NS
Total clearance, ml kg^{-1} h^{-1}	24·46 s.d. 2·52	24·49 s.d. 1·01	NS
Percent of dose in the liver[a]	2·77 s.d. 0·09	2·94 s.d. 0·07	$P < 0.005$
Free fraction in liver[a] × 100	0·148 s.d. 0·019	0·134 s.d. 0·015	NS
Free concentration in the liver[a], ng g^{-1}	0·55 s.d. 0·07	0·59 s.d. 0·05	NS

[a] Estimated 54 h after treatment.

The pharmacokinetic parameters of phenprocoumon in younger and older rats after a single dose of 0·355 mg kg^{-1} are shown in Table 1. The relative liver weight was smaller and the percentage of the dose present in the liver 54 h after dosing was higher in the older rats. All other parameters did not differ.

The analysis of the radioactive material in the liver of younger and older rats 6 h after a single i.v. dose of [^{3}H]vitamin K_1 (64·3 μg kg^{-1}) is shown in Table 2. The fraction of the radioactive dose present as total radioactivity per unit liver weight was not significantly different in younger and older rats. In both groups of rats there was no consistent change of the total activity in the liver after treatment with phenprocoumon. The percentage of the dose associated with vitamin K_1 decreased and that associated with vitamin K_1-epoxide increased after increasing doses of phenprocoumon. This was so in younger and older rats. However, the decrease in vitamin K_1, and especially the increase in vitamin K_1-epoxide, induced by phenprocoumon was less marked in younger than older rats. This resulted in markedly higher vitamin K_1-epoxide/vitamin K_1 ratios in the older rats when compared with those of the younger rats. Treatment with phenprocoumon also enhanced the radioactivity not associated with vitamin K_1 or vitamin K_1-epoxide (i.e. the difference between total activity and that of vitamin K_1 and vitamin K_1-epoxide). The relative increase of this unidentified material was not statistically significant different in both groups.

When the vitamin K_1-epoxide/vitamin K_1 ratios in the liver were plotted as a function of the dose (Fig. 2a) and of the concentration of phenprocoumon

Table 2. Total radioactivity and radioactivity associated with [^{3}H]vitamin K_1 and [^{3}H]vitamin K_1-2,3-epoxide in the livers of younger and older rats. All rats received a single dose of 64·3 μg kg^{-1} [^{3}H]vitamin K_1 and different doses of phenprocoumon. The rats were killed 6 h after injection of vitamin K_1.

		Younger rats[a]				Older rats[b]			
Rat No.	Dose of phenprocoumon mg kg^{-1}	Total radioactivity % of dose per g liver	Vitamin K_1 % of dose per g liver	Vitamin K_1-epoxide % of dose per g liver	Vitamin K_1-epoxide/Vitamin K_1 ratio	Total radioactivity % of dose per g liver	Vitamin K_1 % of dose per g liver	Vitamin K_1-epoxide % of dose per g liver	Vitamin K_1-epoxide/Vitamin K_1 ratio
1	0·00	2·31	1·44	0·20	0·14	2·64	1·81	0·18	0·10
2	0·02	2·13	1·36	0·21	0·15	2·44	1·55	0·19	0·12
3	0·10	1·98	0·82	0·22	0·27	2·77	0·79	0·89	1·06
4	0·20	2·45	0·58	0·83	1·43	2·33	0·35	0·92	2·63
5	0·30	2·28	0·35	0·65	1·86	2·58	0·34	1·20	3·53
6	0·50	2·11	0·29	0·83	2·86	2·02	0·23	1·02	4·43
7	0·50	2·86	0·37	0·93	2·51	2·71	0·25	1·10	4·40
8	0·80	2·46	0·35	0·98	2·80	2·38	0·24	1·04	4·33
9	2·00	2·78	0·33	1·02	3·09	2·47	0·23	0·90	3·91
10	3·00	2·62	0·33	0·90	2·73	2·57	0·24	1·13	4·71
11	3·00	—	—	—	—	2·50	0·19	0·84	4·42
Wilcoxon matched pairs signed rank test						NS	NS	$P < 0.05$	$P < 0.01$

[a] Body weight 289 (14) g; liver weight 8·63 (0·64) g (mean with s.d.).
[b] Body weight 467 (13) g; liver weight 10·94 (0·72) g (mean with s.d.).

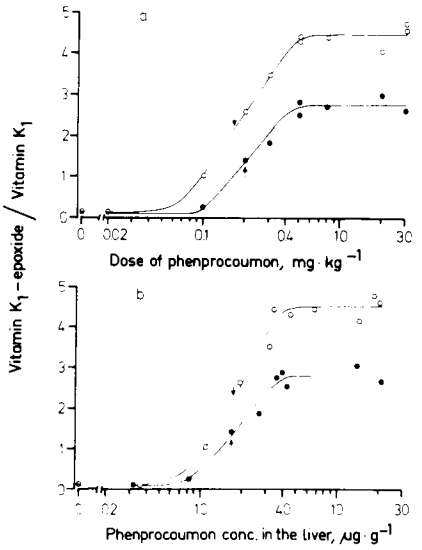

FIG. 2. Effect of different doses of phenprocoumon on the vitamin K_1-epoxide/vitamin K_1 ratio in the liver of younger (●) and older (○) male inbred Lewis rats 6 h following a single i.v.-dose of 64·3 μg kg^{-1} [^{3}H] vitamin K_1. Dose-response (Fig. 2a) and liver concentration–response curve (Fig. 2b). Phenprocoumon was administered intravenously 30 min before the injection of vitamin K_1. Each point represents one rat. Arrows indicate the ED50- and EC50-values, respectively.

in the liver (Fig. 2b), typical dose- and concentration-response curves were obtained. The ED50-values (dose of phenprocoumon which produces a half-maximal response) were 0·165 and 0·200 mg kg^{-1} and the EC50-values (concentration of phenprocoumon in the liver which produces a half-maximal response) were 0·175 and 0·170 μg g^{-1} for older and younger rats, respectively. The maximal response obtained was 1·6-fold higher in the older rats than in the younger rats.

DISCUSSION

A greater anticoagulant response to warfarin of rats in old age has been described by Shepherd et al (1977) and was confirmed in the present stud' for the anticoagulant drug phenprocoumon (Fig. 1) despite the much smaller age difference of our two groups of rats (we used 12 and 36 weeks old rats compared with the 11·5 and 86 weeks old rats studied by Shepherd et al 1977).

Differences in the pharmacokinetics of phenprocoumon are unlikely to account for its greater anticoagulant activity in rats of older age (Table 1). The only difference observed was a slightly higher phenprocoumon concentration in the liver of older rats which results from their lower relative liver weight. For warfarin it was also shown that there are no distinct differences between younger and older rats in the half-life, apparent volume of distribution and plasma clearance of that drug (Shepherd et al 1977).

Thus, we considered it necessary to investigate the effect of phenprocoumon on the metabolism of vitamin K_1 in both groups of rats. Evidence has grown that the hypoprothrombinaemic effect of oral anticoagulant drugs is closely linked to their ability to inhibit the vitamin K_1-epoxide reductase in the liver (Willingham & Matschiner 1974; Ren et al 1977; Whitlon et al 1978). This enzyme reduces the biologically inactive epoxide, which is formed from vitamin K_1 during the carboxylation of 'precursor' clotting factors (Willingham & Matschiner 1974), back to the native vitamin (Bell & Matschiner 1972; Willingham & Matschiner 1974). The inhibition of the vitamin K_1-epoxide reductase by oral anticoagulant drugs leads to an accumulation of the epoxide in the liver (Matschiner et al 1970; Caldwell et al 1974; Sadowski & Suttie 1974) and in the plasma (Shearer et al 1977; Schmidt et al 1979; Park et al 1979) and to a reduced regeneration of vitamin K_1 (Ren et al 1974; Zimmermann & Matschiner 1974; Ren et al 1977).

Our data, shown in Fig. 2, demonstrate a decrease of the vitamin K_1 content and an increase of the vitamin K_1-epoxide content in the liver with increasing doses of phenprocoumon. These changes are consistent with those observed by Caldwell et al (1974) who injected a tracer dose of 50 ng kg^{-1} [^{3}H]vitamin K_1 into control rats and rats pretreated with 1 mg kg^{-1} warfarin. The increase in the vitamin K_1-epoxide/vitamin K_1 ratio was thought to reflect the inhibition of the vitamin K_1-epoxide reductase by anticoagulant drugs (Caldwell et al 1974; Ren et al 1974; Sadowski & Suttie 1974). Marked differences between younger and older rats were found in the effect of phenprocoumon on the vitamin K_1-epoxide/vitamin K_1 ratio. The higher ratios in the older rats when compared with the younger rats are caused by a slightly more marked decrease of the vitamin K_1 content and by a more pronounced increase of the vitamin K_1-epoxide content (Table 2). When the effect of phenprocoumon on these ratios was plotted according to a dose or concentration–response relationship, curves typical of those to be expected for a drug–receptor interaction were obtained (Fig. 2a, 2b). While the ED50- and EC50-values (dose or concentration of phenprocoumon which produces a half-maximal

response) were only slightly different (ED50) or identical (EC50) in younger and older rats, the maximal response obtained was 1·6-times higher in the older rats when compared with the younger rats.

Differences in the accumulation of the vitamin K_1-epoxide in the liver of both groups of rats could reflect differences in the metabolism of vitamin K_1-epoxide to more polar metabolites. This is unlikely, however, since the radioactivity in the liver which is associated with more polar material than vitamin K_1 and vitamin K_1-epoxide (i.e. the difference between the radioactivity associated with vitamin K_1 and vitamin K_1-epoxide and the total radioactivity) was not much different in both groups of rats.

On the other hand, the rate of vitamin K_1-epoxide formation could be different in younger and older rats. However, an inhibition of the vitamin K_1-epoxidase by oral anticoagulant drugs which has been demonstrated to occur in vitro for the structurally related drug warfarin at concentrations much higher than those required for the inhibition of prothrombin synthesis in vivo (Bell & Stark 1976), should lead rather to a decrease of the vitamin K_1-epoxide/vitamin K_1 ratio. Furthermore, the amount of vitamin K_1 available for epoxidation could be different in younger and older rats.

Since the increase of the vitamin K_1-epoxide/vitamin K_1 ratio after treatment with oral anticoagulant drugs is thought to be due to inhibition of the vitamin K_1-epoxide reductase (Caldwell et al 1974; Ren et al 1974; Sadowski & Suttie 1974) our results most likely reflect age-dependent differences in the effect of phenprocoumon on this enzyme. Whether this is due to differences in the accessibility of the enzyme for phenprocoumon, enzyme activity or enzyme concentration remains to be established.

Interestingly, a greatly reduced effect of warfarin on the vitamin K_1-epoxide cycle was also observed in coumarin-resistant rats (Bell & Caldwell 1973; Zimmermann & Matschiner 1974; Bell et al 1976). It seems likely, therefore, that the age-related differences in the anticoagulant response to phenprocoumon are also closely related to the observed quantitative differences in the metabolism of vitamin K_1.

In conclusion, the interruption of the vitamin K_1-epoxide cycle by phenprocoumon is more pronounced in older than in younger rats. This may cause the older rats to react more sensitively to oral anticoagulant drugs than younger rats.

REFERENCES

Bell, R. G., Matschiner, J. T. (1972) Nature (London) 237: 32–33

Bell, R. G., Caldwell, P. T. (1973) Biochemistry 12: 1759–1762

Bell, R. G., Caldwell, P. T., Holm, E. E. T. (1976) Biochem. Pharmacol. 25: 1067–1070

Bell, R. G., Stark, P. (1976) Biochem. Biophys. Res. Commun. 72: 619–625

Caldwell, P. T., Ren, P., Bell, R. G. (1974) Biochem. Pharmacol. 23: 3353–3362

Husted, S., Andreasen, F. (1977) Eur. J. Clin. Pharmacol. 11: 351–358

Levy, G., Lai, C.-M., Yacobi, A. (1978) J. Pharm. Sci. 67: 229–231

Matschiner, J. T., Bell, R. G., Amelotti, J. M., Knauer, T. E. (1970) Biochim. Biophys. Acta 201: 309–315

Park, B. K., Leck, J. B., Wilson, A. C., Serlin, M. J., Breckenridge, A. M. (1979) Biochem. Pharmacol. 28: 1323–1329

Ren, P., Laliberte, R. E., Bell, R. G. (1974) Mol. Pharmacol. 10: 373–380

Ren, P., Stark, P. Y., Johnson, R. L., Bell, R. G. (1977) J. Pharmacol. Exp. Ther. 201: 541–546

Routledge, P. A., Chapman, P. H., Davies, D. M., Rawlins, M. D. (1979) Br. J. Clin. Pharmacol. 8: 243–247

Sadowski, J. A., Suttie, J. W. (1974) Biochemistry 13: 3696–3699

Schmidt, W., Beermann, D., Oesch, F., Jähnchen, E. (1979) J. Pharm. Pharmacol. 31: 490–491

Schmidt, W., Jähnchen, E. (1977) Ibid. 29: 266–271

Schmidt, W., Jähnchen, E. (1979) J. Pharmacokinet. Biopharm. 7: 643–663

Shearer, M. J., McBurney, A., Barkhan, P. (1973) Br. J. Haematol. 24: 471–479

Shearer, M. J., McBurney, A., Breckenridge, A. M., Barkhan, P. (1977) Clin. Sci. Molec. Med. 52: 621–630

Shepherd, A. M. M., Hewick, D. S., Moreland, T. A., Stevenson, I. H. (1977) Br. J. Clin. Pharmacol. 4: 315–320

Tishler, M., Fieser, L. F., Wendler, N. L. (1940) J. Am. Chem. Soc. 62: 2866–2871

Whitlon, D. S., Sadowski, J. A., Suttie, J. W. (1978) Biochemistry 17: 1371–1377

Willingham, A. K., Matschiner, J. T. (1974) Biochem. J. 140: 435–441

Zimmermann, A., Matschiner, J. T. (1974) Biochem. Pharmacol. 23: 1033–1040