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3-METHYLCHOLANTHRENE INDUCTION OF ENZYMES IN THE VITAMIN K-DEPENDENT CARBOXYLATION SYSTEM

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Abstract—The effect of 3-methylcholanthrene on liver enzymes in the vitamin K-dependent carboxylation system has been investigated in normal rats and rats treated with the anticoagulant warfarin. It was found that 3-methylcholanthrene did not interfere with the anticoagulant function of the drug. Treatment of rats with 3-methylcholanthrene resulted in a 2.7-fold increase in liver cytosolic DT-diaphorase activity and a 1.5-fold increase in liver microsomal vitamin K-dependent carboxylase activity. A pathway for production of reduced vitamin K cofactor for the vitamin K-dependent carboxylase is catalyzed by DT-diaphorase and an as yet unidentified NADH-specific dehydrogenase(s). The data suggest that the unidentified enzyme(s) in the pathway is not induced by 3-methylcholanthrene.

Vitamin K-dependent carboxylase is an integral membrane protein of the endoplasmic reticulum [1]. The enzyme is necessary for correct processing of vitamin K-dependent secretory glycoproteins, and the processing step involves γ-carboxylation of specific glutamic acid residues in precursor proteins converting them to γ-carboxyglutamic acid containing proteins [1, 2]. Several of the protein factors of the hemostatic system are vitamin K-dependent proteins, and their synthesis is mainly carried out by the liver [1, 2].

The fully reduced form of vitamin K, vitamin K hydroquinone, is a necessary cofactor for the carboxylase. Concomitant with carboxylation of the protein precursors, the reduced vitamin is converted into vitamin K 2,3-epoxide. The epoxide can be reduced by liver enzymes back to the reduced cofactor form of the vitamin and the enzymes establish a red/ox.-cycle of vitamin K [3, 4]. Vitamin K quinone is an intermediate reduction product in the conversion of the epoxide to the hydroquinone, and the quinone can be reduced by certain liver dehydrogenases which constitute an alternative pathway for vitamin K reduction in the liver [5]. Recently we have shown [5] that the enzymes constituting this pathway in rat liver are (1) DT-diaphorase (EC 1.6.99.2) and (2) an unidentified NADH specific dehydrogenase(s).

Various xenobiotics are powerful inducers of enzymes of the endoplasmic reticulum. However, the enzymes are under different genetic control and the differences are often reflected in the ability a chemical has to induce the enzymes. For instance, 3-methylcholanthrene (3-MC†) is a powerful inducer of DT-diaphorase [6, 7], but phenobarbital is a weak inducer of this enzyme [6, 7]. On the other hand, phenobarbital is a powerful inducer of many proteins of the mixed-function oxidase system [8] and also other well characterized enzymes of the endoplasmic reticulum [9, 10]. Thus, enzyme induction by various xenobiotics has been used to classify enzymes according to their pattern of induction. In this study we have used 3-MC to decide whether or not this chemical induces the unknown dehydrogenase which is involved in vitamin K reduction. We report that the vitamin K-dependent carboxylase was induced by 3-MC. However, in contrast to DT-diaphorase and the carboxylase, the unidentified dehydrogenase appeared to be unaffected by 3-MC.

MATERIALS AND METHODS

Animals. Male Sprague–Dawley rats (150–200 g) were used for the experiments. Rats were divided into four groups (five rats/group). In two groups, rats were injected intraperitoneally on three consecutive days with 3-MC (20 mg/kg) dissolved in corn oil. Each rat within the remaining two groups received the same amount of corn oil per kg. On day 4 each rat within one of the two groups receiving 3-MC and each rat within one of the two groups receiving corn oil were given an intraperitoneal injection of warfarin (30 mg/kg) in saline and were then fasted for 24 hr before being killed. The other two groups not receiving warfarin were given saline as a control.

Preparation of liver microsomes and cytosol. Livers were perfused with ice-cold saline through the portal vein. After perfusion, all livers from rats within each individual group were pooled and homogenized as described by Suttie *et al.* [11] in 250 mM sucrose, 25 mM imidazole and 1 mM VAPONA, pH 7.2 (SI-VAPONA buffer). The homogenate was centrifuged twice for 10 min at 10,000 *g*, and the last supernatant fraction was subjected to centrifugation

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† Abbreviations: CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate; 3-MC, 3-methylcholanthrene; and vitamin K_1H_2, vitamin K hydroquinone.

at 100,000 *g* for 60 min. The supernatant fraction from the 100,000 *g* spin of liver cytosol was removed and stored frozen at −20° until used for measurement of DT-diaphorase activity. The microsomal pellets were stored in liquid N_2 with no detectable loss in carboxylase activity.

Enzyme activities. Vitamin K-dependent carboxylase activity was measured in microsomes solubilized with the detergent Triton X-100. The solubilization buffer was 250 mM sucrose, 500 mM KCl, 1.5% Triton X-100, 1 mM VAPONA and 25 mM imidazole, pH 7.2 (SIK-VAPONA buffer). Carboxylase activity was measured as $^{14}CO_2$ incorporation into the synthetic peptide substrate Phe-Leu-Glu-Glu-Leu as described by Esmon and Suttie [12]. Incubations were carried out for 30 min at 25° [5] and contained either (1) chemically reduced vitamin K_1H_2 (100 μg/ml) to trigger carboxylase activity or (2) [vitamin K_1 (100 μg/ml) + NADH (2 mM)] to trigger carboxylase activity when supported by the dehydrogenase pathway for vitamin K reduction [4]. The commercially available vitamin K_1 preparation Aquamephyton was used in the assay with NADH. DT-diaphorase activity was measured at room temperature as described by Dallner [13] with 2,6-dichlorophenol indophenol (DCPIP) as the electron acceptor. The diaphorase activity that was inhibited with 100 μM dicumarol was used to calculate DT-diaphorase activity. Prothrombin precursor activity was measured in detergent extracts of microsomes after *Echis carinatus* activation as described by Nelsestuen and Suttie [14]. The extracts were the supernatant fractions obtained after suspending the microsomes in 25 mM imidazole and 0.5% CHAPS, pH 7.2, as we have described previously [15]. Prothrombin precursor activity was determined as NIH units by comparing clotting times to a thrombin standard (Thrombostat).

Affinity chromatography. DT-diaphorase was removed from solubilized microsomes by immunoaffinity chromatography on a column of anti-DT-diaphorase-IgG-Sepharose equilibrated and eluted in 25 mM imidazole, 500 mM NaCl and 0.2% Triton X-100, pH 7.2, as we have described previously [5].

Materials. Vitamin K_1 (Sigma) was reduced to vitamin K_1 hydroquinone as described by Sadowski *et al.* [16]. Aquamephyton (vitamin K_1; 10 mg/ml) was obtained from Merck, Sharp & Dohme. The pentapeptide Phe-Leu-Glu-Glu-Leu was purchased from Vega Fox Biochemicals (Tucson, AZ). $NaH^{14}CO_2$ (60 mCi/mmol) was from the Amersham Corp. CHAPS, Triton X-100, and 3-MC were from Sigma. The insecticide VAPONA (2,2-dichlorovinyl phosphate) was a gift from the Shell Oil Co. (Houston, TX). All other chemicals were of reagent grade or better. Protein was measured with the Bio-Rad Protein assay.

RESULTS AND DISCUSSION

As expected, treatment of rats with 3-MC resulted in enhanced liver DT-diaphorase activity. The specific activity of this enzyme in liver cytosol from 3-MC-treated and warfarin + 3-MC-treated rats was 2.7-fold higher than the activity measured in the group of rats that had received corn oil control or corn oil plus warfarin (warfarin-treated) (Table 1). There was no significant difference in specific DT-diaphorase activity between rats receiving corn oil and corn oil plus warfarin, confirming earlier studies showing that warfarin has no inhibitory effect on DT-diaphorase *in vivo* [15].

In rat liver, warfarin results in accumulation of vitamin K-dependent precursor proteins [17, 18], and the degree of anticoagulation caused by the drug can be evaluated from the quantity of precursors present in the liver. To decide whether or not 3-MC interfered with the anticoagulant effect of warfarin, we measured prothrombin precursor activity in microsomes from the pooled livers. Thrombin activity released from these precursors after *E. carinatus* activation is shown in Table 1. Corn oil plus warfarin treatment of rats resulted in a 4.3-fold increase in precursor activity over the activity measured in corn oil treated rats (Table 1). However, there was no difference in precursor activity between the corn oil plus warfarin treated group and the group of rats treated with warfarin and 3-MC (Table 1). Treatment with 3-MC without warfarin had only a small effect on the prothrombin precursor activity (Table 1). This suggests that 3-MC does not interfere with the anticoagulant function of warfarin. Thus, any additional effect on the vitamin K-dependent carboxylation system observed in the group of rats

Table 1. Liver DT-diaphorase and prothrombin precursor activities in warfarin and 3-methylcholanthrene treated rats

Treatment	DT-diaphorase activity (nmol/mg/min)	Prothrombin precursor activity (NIH units/mg)
Control	270 ± 17	0.344 ± 0.026
Warfarin	282 ± 14	1.48 ± 0.14
3-MC	746 ± 19	0.421 ± 0.038
Warfarin + 3-MC	764 ± 21	1.55 ± 0.33

Four groups of rats, each containing five rats, were injected with corn oil (control), 3-MC, warfarin, and warfarin plus 3-MC according to the protocol described in Materials and Methods. Liver cytosol and microsomes were prepared, as described, from pooled livers in each group. DT-diaphorase activity was measured as DCPIP reduction in liver cytosol and prothrombin precursor activity as thrombin activity in 0.5% CHAPS extracts of liver microsomes (see Materials and Methods). Each number is the average activity calculated from three parallel measurements ± standard deviations.

Table 2. Carboxylase activity in microsomes from rats treated with warfarin and 3-methylcholanthrene

Treatment	Carboxylase activity (cpm/mg × 10^{-3})					
	Preparation A			Preparation B		
	K_1H_2	K_1 + NADH	$\frac{K_1H_2}{K_1 + NADH}$	K_1H_2	K_1 + NADH	$\frac{K_1H_2}{K_1 + NADH}$
Control	2.3 ± 0.21	1.38 ± 0.16	1.7	7.21 ± 0.36	0.59 ± 0.08	12.2
3-MC	13.6 ± 0.8	8.50 ± 0.31	1.6	33.8 ± 1.8	1.62 ± 0.18	20.9
Warfarin	28.8 ± 1.3	15.1 ± 1.0	1.9	67.7 ± 3.6	5.13 ± 0.45	13.2
Warfarin + 3-MC	40.1 ± 2.4	24.8 ± 1.7	1.6	102.4 ± 7.0	5.30 ± 0.50	19.3

Four groups of rats, each containing five rats, were injected with corn oil (control), 3-MC, warfarin, and warfarin plus 3-MC according to the protocol described in Materials and Methods. Liver microsomes were prepared from pooled livers from each group, and the microsomes were solubilized with the detergent Triton X-100 as described (Preparation A). In order to specifically remove DT-diaphorase, solubilized microsomes were also immunoabsorbed on a column of anti-DT-diaphorase-IgG-Sepharose (see Materials and Methods) (Preparation B). Vitamin K-dependent carboxylase activity was measured in Preparations A and B when supported by (vitamin K_1 + NADH) (K_1 + NADH) and chemically reduced vitamin K_1H_2 (K_1H_2) respectively. The numbers are the average activities determined from three parallel incubations ± standard deviations.

treated with warfarin + 3-MC could not be the result of anticoagulation of the rats by 3-MC.

Table 2 (Preparation A) lists carboxylase activities measured in liver microsomes from the different groups of rats when the activity was supported by the dehydrogenase pathway (vitamin K_1 + NADH) and chemically reduced vitamin K_1H_2 respectively. There was a marked increase in carboxylase activity in warfarin treated and 3-MC treated rats (Table 2). It appears from our data that warfarin and 3-MC have additive effects on the level of carboxylase activity in rat liver (Table 2). The ratio between the two differently supported activities is also shown (Table 2). This ratio was similar for all four groups of rats used in the study (1.6 to 1.9; Table 2) which suggested that induction of enzymes of the dehydrogenase pathway had also occurred as a result of the 3-MC treatment. As shown in Table 1, the pathway I enzyme DT-diaphorase was indeed induced in livers from 3-MC treated rats. When prepared according to the procedure used in this study, the microsomal vitamin K-dependent carboxylation system does contain DT-diaphorase [5], and this enzyme has been shown to be induced in microsomes by 3-MC in a manner parallel to the cytosolic enzyme [19]. However, the question remained whether or not the second enzyme(s) in the pathway was induced. In contrast to DT-diaphorase, no specific test system is available for this enzyme(s). The enzyme could, however, provide the carboxylase with reduced vitamin K_1H_2 cofactor and we, therefore, utilized the ability of the enzyme to trigger carboxylase activity as a way to evaluate its activity in Triton X-100 solubilized microsomes. For this, we removed DT-diaphorase from solubilized microsomes by affinity chromatography [5] and created a test system where the unidentified enzyme(s) was responsible for quinone reduction by the dehydrogenase pathway. Carboxylase activities measured in solubilized microsomes prepared from the four groups of rats after passage through the anti-DT-diaphorase immunoaffinity column are shown in Table 2 (Preparation B). There was even a greater difference in vitamin K_1H_2 supported carboxylase activity between warfarin treated and warfarin plus 3-MC treated rats after the test system had passed the affinity column and the specific activity of the carboxylase was enhanced (Table 2, Preparation B). On the other hand, immunoabsorption resulted in significantly lower (vitamin K_1 + NADH) supported carboxylase activities (Table 2). Importantly, the (vitamin K_1 + NADH) supported activity measured in immunoabsorbed microsomes from warfarin plus 3-MC treated rats was not significantly different from the same activity measured in immunoabsorbed microsomes from warfarin treated rats (Table 2; Preparation B). Since the carboxylase activity was greatly enhanced in immunoabsorbed microsomes from warfarin plus 3-MC treated rats, the ratio (vitamin K_1H_2)/(vitamin K_1 + NADH) rose from 13.2 in immunoabsorbed microsomes from warfarin treated rats to 19.3 in immunoabsorbed microsomes from warfarin plus 3-MC treated rats. We repeated these experiments three times with new groups of rats. From three independent experiments the averages of the above ratios were calculated to be 12.9 ± 0.4 and 19.0 ± 0.3. The numbers are presented with standard errors of the mean. The carboxylase activity measurements after immunoabsorption of corn oil treated and corn oil plus 3-MC-treated rats also suggest that the activity of the unknown dehydrogenase was not enhanced by 3-MC treatment of the rats. The ratio rose from 12.2 in corn oil treated rats to 20.9 in corn oil plus 3-MC-treated rats. Since, in our system, production of reduced vitamin K_1H_2 cofactor by the dehydrogenase pathway is clearly the limiting step leading to carboxylation of the pentapeptide, our data show that the activity of the second enzyme(s) cannot be affected significantly by 3-MC treatment of rats.

Thus, 3-MC does not appear to be an inducer of the unidentified vitamin K quinone reductase(s), and this result classifies this enzyme(s) among other microsomal enzymes that also are not induced by 3-MC. 3-MC clearly induced DT-diaphorase. We also demonstrate for the first time that the vitamin K-dependent carboxylase can be induced by xenobiotics. Thus, the carboxylase can be added to the list of microsomal enzymes that are induced by 3-MC. The ratio between vitamin K_1H_2 and [vitamin K_1 + NADH] supported carboxylation on which this conclusion is based has also been used to decide that cytochrome P-450 reductase and cytochrome B reductase are not identical to the unknown enzyme(s) of the dehydrogenase pathway [5]. In those studies, the ratio did not change in solubilized microsomes after chromatography on dehydrogenase specific affinity resins. This observation supports our data. It appears that a change in the ratio will reflect a change in the enzyme activities supporting vitamin K-dependent carboxylation.

Sadowski *et al.* [20] have reported data which suggest that phenobarbital may have an inducing effect on the vitamin K-dependent carboxylation system in rat liver. Carboxylase activity supported by chemically reduced vitamin K_1H_2 was not measured by Sadowski *et al.* [20], and an effect of phenobarbital on the vitamin K-dependent carboxylase was, therefore, not investigated. Our data suggest that the vitamin K-dependent carboxylase can be induced by xenobiotics.

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