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DEPRESSION OF LIVER MICROSOMAL VITAMIN K EPOXIDE REDUCTASE ACTIVITY ASSOCIATED WITH ANTIBIOTIC-INDUCED COAGULOPATHY

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Abstract—Hypoprothrombinemic changes in blood coagulation parameters, such as prolongation of prothrombin time, increase in the level of plasma protein induced by vitamin K absence, and decrease in plasma prothrombin level, were detected in rats fed a vitamin K-deficient diet. These changes were enhanced by the administration of β-lactam antibiotics containing *N*-methyltetrazolethiol, thiadiazolethiol or methyl-thiadiazolethiol. Microsomal vitamin K epoxide reductase activity was suppressed with the maximum effect at 1–2 days after the treatment and with recovery, thereafter, gradually to the normal level after 5–7 days. Hypoprothrombinemic alterations in blood coagulation parameters following a single administration of antibiotic to vitamin K-deficient rats were somewhat delayed compared with the change in the epoxide reductase activity, but the effects of the antibiotic on both blood coagulation parameters and the enzyme activity disappeared completely 7 days after the antibiotic treatment. Antibiotic-induced depression of the epoxide reductase activity was observed even in the vitamin K sufficient rats, although the hypoprothrombinemic changes in the blood coagulation parameters did not develop. Vitamin K administration could normalize the blood coagulation parameters in the hypoprothrombinemic rats caused by treatment with the antibiotics but without recovery of the decreased epoxide reductase activity. These results suggest that some antibiotics inhibit liver microsomal vitamin K epoxide reductase, which causes hypoprothrombinemia to develop under vitamin K-deficient conditions.

Vitamin K metabolism is required for postribosomal conversion of Glu† to Gla residues in the N-terminal region of vitamin K-dependent coagulation factors II, VII, IX and X [1, 2]. Three enzymes are involved in vitamin K metabolism and clotting factor synthesis (Fig. 1). γ-Glutamylcarboxylase plays an essential role in the synthesis of vitamin K-dependent clotting factors associated with the conversion of the hydroquinone form of vitamin K to vitamin K epoxide. Vitamin K quinone reductase (K reductase) is needed for the carboxylase to complete its action catalyzing the conversion of the quinone form of vitamin K to the hydroquinone form, since vitamin K is supplied in an ineffective quinone form. Vitamin K epoxide reductase (epoxide reductase), which functions to regenerate this vitamin from the epoxide, is known to be very sensitive to inhibition by coumarin anticoagulants and is believed to be the target of their anticoagulant effect [2–5].

Recent clinical work has shown that several β-lactam antibiotics, having an *N*-methyltetrazolylthiomethyl group at the 3-position of the cephem nucleus, cause vitamin K-reversible hypoprothrombinemia [6–8]. Subsequently, Bechtold *et al.* [9] reported a transient increase in the plasma concentration of vitamin K epoxide when subjects who had been pretreated with NMTT-containing antibiotics were given vitamin K. Administration of NMTT-containing antibiotics or NMTT itself to vitamin K-deficient rats causes them to develop hypoprothrombinemia concomitant with a depression of DTT-dependent vitamin K epoxide reductase activity in liver microsomes, although γ-carboxylase

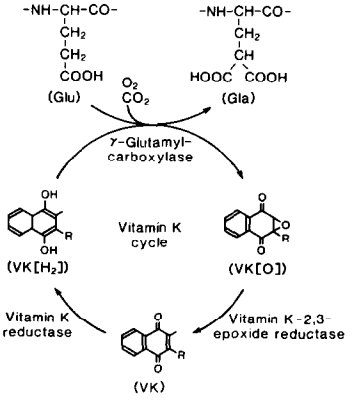

Fig. 1. Vitamin K cycle and γ-glutamylcarboxylase. Hydroquinone and quinone forms of vitamin K are shown in the figure as VK[H_2] and VK respectively. VK[O] represents vitamin K-2,3-epoxide.

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† Abbreviations: Glu, glutamic acid; Gla, γ-carboxyglutamic acid; PT, prothrombin time; APTT, activated partial thromboplastin time; PIVKA, protein induced by vitamin K absence (descarboxyprothrombin); DTT, dithiothreitol; LMOX, latamoxef; NMTT, *N*-methyltetrazolethiol (1-methyl-1*H*-tetrazole-5-thiol); DATT, 1-(2-dimethylamino)ethyl-1*H*-tetrazole-5-thiol; MTDT, 2-methyl-1,3,4-thiadiazole-5-thiol; and TDT, 1,3,4-thiadiazole-5-thiol.

and NADH-dependent vitamin K reductase activities are not inhibited [10–12]. These results suggested that the antibiotic-induced hypoprothrombinemia that develops under vitamin K-deficient conditions results from inactivation of the liver vitamin K epoxide reductase cased by antibiotics.

In the present study, we examined the *in vivo* effects of various β-lactam antibiotics on the blood coagulation parameters and liver microsomal epoxide reductase. Administration of these antibiotics caused inhibition of the epoxide reductase activity in both vitamin K-sufficient and -deficient rats, although the hypoprothrombinemic changes in blood coagulation parameters were detected only in the vitamin K-deficient state.

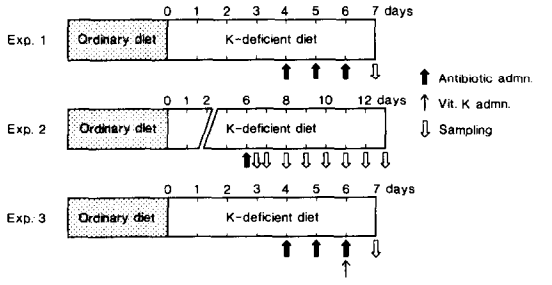

Fig. 2. Experimental schedule for animal treatments. Rats fed a vitamin K-deficient diet were given antibiotics or vitamin K as indicated in the figure. Closed and open arrows represent administration of antibiotics and sampling points respectively. The point of vitamin K administration is also shown.

MATERIALS AND METHODS

Animals. Jcl:Sprague–Dawley strain male rats (9–10 weeks old) were used. Animals were kept in an air-conditioned room (25 ± 1°, 50–60% humidity) lighted 12 hr a day (8:00 a.m. to 8:00 p.m.) and maintained on an ordinary diet (CA-1, Clea Japan, Inc., Tokyo) and water *ad lib.* When vitamin K-deficient rats were used for the experiments, animals were maintained on a vitamin K-deficient diet, prepared in our laboratories, which contained vitamin K-free casein (18.0%), sucrose (67.6%), fat (a mixture of rapeseed and soybean oils) (8.0%), a salt mixture (Hegsted salt) (4.0%), fibers (1.5%), and adequate amounts of vitamins, except for vitamin K. The salt mixture was prepared as described by Hegsted *et al.* [13]. The fat and fibers used were salad oil purchased from the Hohnen Oil Co. (Tokyo) and cellulose powder from the Asahi Chemical Industry Co. (Osaka), respectively. The animals were kept on a suspended wire-bottomed cage to prevent coprophagy. Vitamin K contents in the ordinary and deficient diet were about 500 and 30–40 ng/g, respectively.

Antibiotics and chemicals. β-Lactam antibiotics used for the experiments were obtained as follows: LMOX, cefamandole and cephalothin from the Shionogi & Co. (Osaka), cefmenoxime and cefotiam from Takeda Chemical Industries (Osaka), cefoperazone from the Toyama Chemical Co. (Tokyo), cefmetazole from the Sankyo Co. (Tokyo), cefazolin and ceftezole from the Fujisawa Pharmaceutical Co. (Osaka), and cefotetan from the Yamanouchi Pharmaceutical Co. (Tokyo). The substrate for the enzyme reaction, vitamin K epoxide (menaquinone-4 2,3-epoxide), was synthesized in our laboratories. We prepared both phylloquinone and menaquinone derivatives, but the phylloquinone derivative was hard to crystallize. Thus, menaquinone epoxide was used as the substrate throughout the experiment. Other chemicals of the purest grade available were obtained commercially and used for the experiments without further purification.

Determination of blood coagulation parameters. Antibiotics were dissolved in distilled water and injected intravenously at a volume of 1.0 ml/kg. In some experiments, vitamin K (phylloquinone) was administered subcutaneously. Figure 2 shows the experimental schedule for the treatment of animals and the sampling of blood and liver. The animals were placed under ether anesthesia to obtain the samples. Blood samples were withdrawn from the vena cava with a disposable syringe containing 1/10 volume of 3.8% sodium citrate solution. After obtaining the plasma by centrifugation, PT and APTT were assayed with COAG-A-MATE-X2 (Warner Lambert Co., Morris Plains, NJ). Plasma prothrombin and PIVKA were assayed as reported previously [14].

Determination of vitamin K epoxide reductase activity. To obtain liver microsomes for the activity determination, liver samples were homogenized in ice-cold 0.25 M sucrose containing 50 mM Tris–HCl buffer (pH 7.4), and the microsomal fraction was obtained by differential centrifugation as described previously [15]. Microsomal pellets were stored at −70° under nitrogen atmosphere until the activity determination (within 1 week), without any loss of the activity. The protein concentration of the sample was determined by the method of Lowry *et al.* [16] using bovine serum albumin as a standard.

Microsomal K epoxide reductase activity was determined essentially as described previously [3] with slight modifications. One milliliter of reaction mixture contained microsomes (0.3 to 0.5 mg protein), 20 μM substrate (menaquinone-4 2,3-epoxide), 0.5 mM DTT, 0.15 M KCl and 0.2 M Tris–HCl buffer (pH 7.4). The reaction was initiated by adding DTT. After a 5-min incubation at 25° with moderate shaking, the reaction was terminated by mixing 2 ml of a mixture of isopropanol and hexane (3:2, v/v) on a Thermomics mixer (Thermomics Co., Tokyo) for about 10–15 sec. After centrifugation, 0.5 ml of the hexane layer was removed, and the solvent was allowed to evaporate under nitrogen atmosphere. The resulting residue was redissolved in 0.2 ml of isopropanol. All these procedures were carried out in a dark room under an orange light. A portion of this extract (80 μl) was analyzed by HPLC using an instrument equipped with a Waters model 6000A pump, a Reodyne 7125 injector, a Shimadzu SPD-2A detector (set at 254 nm) and a Shimadzu C-R1B chromatopac. The column was Cosmosil $5C_{18}$ (4.6 mm i.d. × 150 mm) with a MPLC guard column (4 mm i.d. × 30 mm, Brownlee Lab.). The mobile phase used was a mixture of methanol and water (99:1, v/v) at a flow rate of 1.0 ml/min. Under these

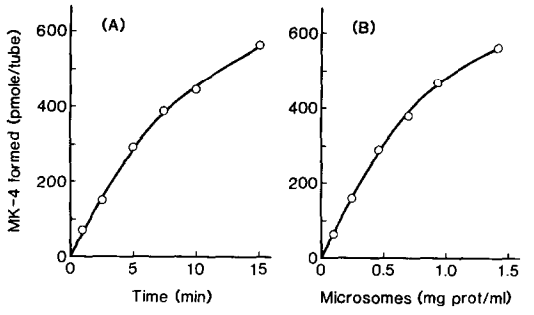

Fig. 3. Formation of vitamin K by microsomal vitamin K epoxide reductase. Enzymatic conversion of menaquinone-4 2,3-epoxide was measured by determining the reaction product (MK-4). (A) Time course in the enzymatic reaction. Reaction mixture (1 ml) containing 0.47 mg protein of microsomes and 20 μM substrate (menaquinone-4 2,3-epoxide) was incubated at 25° for various times as indicated in the figure. (B) Correlation between microsomal protein and vitamin K formation. The reaction mixture containing various concentrations of microsomes was incubated at 25° for 5 min.

analytical conditions, the hydroquinone, epoxide, and quinone forms of menaquinone-4 were detected with t_R values of 2.9–3.9, 7.4–7.7 and 10.5–11.0 respectively. The minimum amount of detectable menaquinone-4 was about 4 pmol/injection. The epoxide reductase activity was determined by calculating the amount of vitamin K (menaquinone-4) formed, and expressed as the amount of vitamin K quinone formed per minute per milligram of microsomal protein.

RESULTS

Assay of microsomal vitamin K epoxide reductase activity. Under the analytical conditions employed, enzymatic conversion of the K epoxide (menaquinone-4 2,3-epoxide) to the quinone form of vitamin K (menaquinone-4) was detected by an HPLC technique. The HPLC chromatogram of the reaction mixture showed a single peak (quinone form) as the product, and the DTT adduct reported by Hildebrandt *et al.* [17] and Thijssen *et al.* [18] was not detected in our assay system. The reaction proceeded linearly for about 5 min, then the reaction rate decreased during a long incubation (Fig. 3A). When lower concentrations of microsomes were added to the reaction mixture (less than 0.7 mg protein/ml), a close correlation was obtained between the microsomal concentration and the formation of the reaction product (Fig. 3B). When the epoxide reductase activity was measured using various reductants, higher activities were detected by the addition of DTT [19]; thus, we used DTT as the reductant. Since solubilization of microsomal enzyme with detergent caused only slight activation of the activity, intact microsomes were used for the activity determination. From these results, we finally established the assay system as described in Materials and Methods.

Under the assay conditions employed, the enzyme activity was determined using liver microsomes obtained from rats of various states. Feeding of vitamin K-deficient diet for 4 or 7 days tended to increase the epoxide reductase activity associated with the hypoprothrombinemic changes in blood coagulation parameters (prolongation of PT and APTT, decrease in plasma prothrombin level, and increase in plasma PIVKA level), as shown in Table 1. Fasting of rats for 24 hr caused a slight decrease in the activity, whereas the administration of a typical anticoagulant, warfarin, to the vitamin K-sufficient rats caused a marked decrease in microsomal epoxide reductase activity concomitant with the hypoprothrombinemic changes in the blood coagulation parameters. The results shown in the table suggest that physiological vitamin K-deficiency, but not the drug-induced vitamin K-deficiency, causes an increase in the epoxide reductase activity although the increasing rate was variable, and the characteristic effect of drugs on microsomal epoxide reductase activity was detectable using our assay conditions.

Effect of latamoxef on vitamin K epoxide reductase activity and blood coagulation parameters. Using LMOX as a model of NMTT-containing antibiotics, we studied the *in vivo* effects of β-lactam antibiotics on liver microsomal epoxide reductase and blood coagulation parameters in rats fed an ordinary and vitamin K-deficient diet. Multiple administrations of LMOX to the vitamin K-sufficient rats (ordinary diet fed rats) did not cause hypoprothrombinemic changes in blood coagulation parameters, but the epoxide reductase activity decreased significantly (Table 2). When vitamin K-deficient rats received LMOX, the enzyme activity decreased markedly, and the blood coagulation parameters tended to change to the hypoprothrombinemic values.

Figure 4 shows the microsomal epoxide reductase activity in LMOX-treated rats. When vitamin K-deficient rats were treated with LMOX for 1–3 days, the liver enzyme activity decreased gradually with the increase of administration time. Dose-dependency in the inhibitory action of LMOX was also detected in the K-deficient rats. Blood coagulation parameters in the LMOX-treated rats showed hypoprothrombinemic values concomitant with the decrease in the epoxide reductase activity (data not shown). These results indicate that the effect of β-lactam antibiotics on liver microsomal epoxide reductase activity can be detected with the administration of antibiotics at a dose of at least 300 mg/kg/day, once daily for 3 days. Injection of higher doses of antibiotics caused marked inhibition of the epoxide reductase activity even after a single treatment.

Time course of the effect of LMOX on blood coagulation parameters and vitamin K epoxide reductase. To confirm the participation of microsomal epoxide reductase activity in the development of antibiotic-induced hypoprothrombinemia, alterations of blood coagulation parameters and microsomal enzyme activity in vitamin K-deficient rats were determined periodically following LMOX administration. As shown in Fig. 5A, the epoxide reductase activity decreased gradually with time following the administration of a high dose (1000 mg/kg) of LMOX, and the lowest activity was observed 1–2 days after the administration. Thereafter, the activity recovered gradually, attaining the control

Table 1. Effects of warfarin and vitamin K-deficient diet feeding on liver microsomal vitamin K epoxide reductase activity and blood coagulation parameters in rats

Exptl group		No. of rats	PT (sec)	APTT (sec)	Prothrombin (units/ml)	PIVKA (units/ml)	Vit. K epoxide reductase (pmol/min/mg protein)
1	Ordinary diet	5	10.8 ± 0.04	17.9 ± 0.3	173.4 ± 3.2	1.6 ± 0.2	120.3 ± 13.1
	Vit. K-deficient diet						
	4 days	5	12.4 ± 0.3*	22.3 ± 1.0*	74.1 ± 8.4†	2.9 ± 0.2†	142.1 ± 8.2
	7 days	5	15.0 ± 0.8†	26.6 ± 1.4†	44.1 ± 5.9†	3.8 ± 0.2†	135.6 ± 8.9
2	Control	4					127.6 ± 7.8
	Fasting (24 hr)	4					115.0 ± 2.3*
3	Ordinary diet	4	10.3 ± 0.1	17.1 ± 0.5	174.6 ± 3.2	3.2 ± 0.3	116.5 ± 10.3
	Warfarin	4	20.0 ± 0.7†	30.3 ± 1.2†	32.4 ± 2.0†	8.6 ± 0.3†	1.5 ± 0.7†

Warfarin was given subcutaneously at 1 mg/kg, and liver and blood samples were obtained 24 hr after the treatment. Values are means ± SE.
† Statistically significant ($P < 0.05$ and †$P < 0.01$) against the corresponding control (ordinary diet group).

level 5–7 days later. LMOX-induced alterations in the blood coagulation parameters were somewhat delayed compared with the change in microsomal epoxide reductase activity. PT and plasma levels of prothrombin and PIVKA changed gradually with feeding of the K-deficient diet (Fig. 5, B–D, closed symbols), and further hypoprothrombinemic alterations of these coagulation parameters were detected in rats treated with LMOX. However, the hypoprothrombinemic effect of LMOX disappeared almost completely 7 days after the LMOX treatment (Fig. 5, B–D, open symbols). The results indicate a close correlation of microsomal epoxide reductase with the antibiotic-induced hypoprothrombinemia under vitamin K-deficient states, and the LMOX-related enhancement in the abnormalities of the blood coagulation parameters disappeared following normalization of the epoxide reductase activity.

Comparison of inhibitory action of β-lactam antibiotics on microsomal vitamin K epoxide reductase. Under the experimental conditions employed to detect the LMOX-induced hypoprothrombinemia and the depression of microsomal epoxide reductase, we compared the effects of various β-lactam antibiotics on microsomal enzyme activity in vitamin K-deficient rats. LMOX was used in every experimental group to compensate for variation in the experimental results. Antibiotics employed for the experiments contained heterocyclic thiol compounds at the 3′-position of the cephem nucleus, except that cephalothin does not have the heterocyclic thiol substituent. Chemical structures of these heterocyclic thiol compounds are shown in Fig. 6. Liver enzyme activity in rats treated with a 300 mg/kg/day dose of NMTT-containing antibiotics for 3 days was 20–30% lower than that of the control (Table 3). On the other hand, cefotiam, containing the tetrazole derivative DATT as the 3′-position substituent, exhibited no inhibitory action. Interestingly, MTDT-containing cefazolin and TDT-containing ceftezole showed the strongest inhibitory action among the antibiotics examined, while cephalothin administration caused no inhibition. The results shown in Table 3 suggest that the chemical structure of the 3′-position substituents is closely correlated with the inhibitory action of antibiotics on microsomal epoxide reductase.

Effect of vitamin K on LMOX-induced hypoprothrombinemia and vitamin K epoxide reductase activity. As described above, antibiotic-induced hypoprothrombinemia developed only in the vitamin K-deficient rats. We next examined the effect of vitamin K administration on the LMOX-induced hypoprothrombinemia in these rats. Feeding of the vitamin K-deficient diet caused hypoprothrombinemic changes in blood coagulation parameters which were enhanced by LMOX administration (Table 4). After the development of LMOX-induced hypoprothrombinemia, vitamin K was administered subcutaneously according to the schedule in Fig. 2 (Exp. 3). This was found to normalize blood coagulation parameters, but did not lead to recovery of the epoxide reductase activity. Vitamin K was efficacious in the LMOX-induced hypoprothrombinemic rats by both subcutaneous and oral routes (data not shown).

Table 2. Effect of latamoxef administration on vitamin K epoxide reductase activity and blood coagulation parameters in rats

Exptl group		PT (sec)	APTT (sec)	Prothrombin (units/ml)	PIVKA (units/ml)	Vit. K epoxide reductase (pmol/min/mg protein)
1	Ordinary diet	11.2 ± 0.2	19.1 ± 0.6	179.4 ± 2.8	2.1 ± 0.1	144.5 ± 2.4
	+LMOX 3 days	11.4 ± 0.4	17.0 ± 0.4	185.7 ± 16.1	2.7 ± 0.3	105.8 ± 11.4*
	+LMOX 7 days	11.0 ± 0.2	17.9 ± 0.3	198.6 ± 7.5	2.8 ± 0.3	104.7 ± 1.5†
2	Vit. K-deficient diet	13.8 ± 1.3	23.4 ± 2.4	91.1 ± 32.7	3.9 ± 0.4	165.1 ± 4.8
	+LMOX 3 days	15.2 ± 1.2	26.2 ± 2.4	66.6 ± 21.5	5.0 ± 0.2	114.5 ± 3.8†

Animals in experimental group 2 were treated as shown in Fig. 1 (Exp. 1). Latamoxef (LMOX) was given intravenously at 300 mg/kg/day. Values are means ± SE of four animals.
† Statistically significant ($P < 0.05$ and † $P < 0.01$) against the corresponding control.

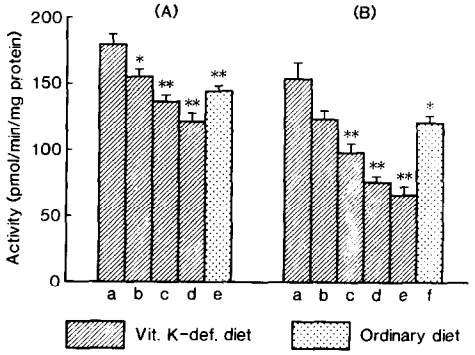

Fig. 4. Effect of latamoxef (LMOX) administration on liver microsomal vitamin K epoxide reductase activity. In experimental group (A), animals in group (a) were the vitamin K-deficient control, and the K-deficient rats in groups (b)–(d) were treated as shown in Fig. 2 (Exp. 1). LMOX was administered intravenously for (b) 1 day (day 6), (c) 2 days (days 5 and 6) and (d) 3 days (days 4–6) at 300 mg/kg/day. In experimental group (B), the K-deficient rats were treated with LMOX for 3 days as shown in Fig. 2 (Exp. 1). Doses of LMOX were (a) 0 (control), (b) 100, (c) 300, (d) 600 and (e) 1000 mg/kg/day. Enzyme activity in the ordinary diet-fed rats ("e" and "f" in experimental groups (A) and (B) respectively) is shown in the figure for comparison. Values are means ± SE of four rats. Key: (*) and (**) statistically significant ($P < 0.05$ and $P < 0.01$ respectively) against the control (group a).

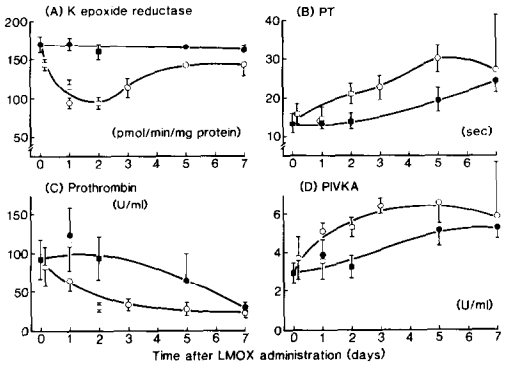

Fig. 5. Time course in the LMOX-induced changes of microsomal epoxide reductase activity and blood coagulation parameters in vitamin K-deficient rats. Animal treatment and blood and liver sampling were carried out as shown in Fig. 2 (Exp. 2). Vitamin K-deficient rats were given a single intravenous injection of LMOX at 1000 mg/kg and then maintained on the vitamin K-deficient diet. Liver and blood samples were obtained 4 hr to 7 days after the LMOX administration. Experiments were performed twice, and the results from both experiments are shown together in the figure. Open and closed symbols represent LMOX-treated rats and control rats (vitamin K-deficient diet alone) respectively. Circles and squares in the figure represent different experiments. Values are the means ± SE of four animals.

Effect of multiple administration of LMOX on vitamin K epoxide reductase activity in liver microsomes. As described in Fig. 5, the effect of a single administration of a high dose of LMOX on the epoxide reductase activity in the K-deficient rats was long-lasting, with the maximal effects at 1–2 days after the treatment. When K-sufficient rats received a single intravenous administration of LMOX at 1000 mk/kg, the lowest activity of the epoxide reductase was detected 1–3 days after the treatment. The activity then recovered gradually, reaching normal levels 5–7 days later (Fig. 7). When animals fed the ordinary diet received multiple administrations of LMOX for 3 and 7 days, a marked decrease in the enzyme activity was observed 30 min after the last administration and the lowest activity was detected continuously for 1–2 days after the last administration. Return of the enzyme activity followed approximately the same time schedule as that for the

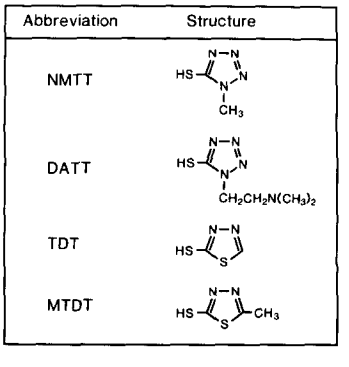

Fig. 6. Chemical structures of heterocyclic thiol compounds, which are 3'-position substituents of various β-lactam antibiotics.

Table 3. Effects of various antibiotics on microsomal vitamin K epoxide reductase activity

Exptl group	Treatment of rats	3′-Position substituent	Vit. K epoxide reductase (pmol/min/mg protein)	
1	Control		162.2 ± 7.2	(100)
	Latamoxef	NMTT	115.4 ± 6.7*	(71.1)
	Cefoperazone	NMTT	137.5 ± 1.6†	(84.8)
	Cefamandole	NMTT	123.0 ± 2.9*	(75.8)
2	Control		177.8 ± 8.3	(100)
	Latamoxef	NMTT	142.8 ± 5.6†	(80.3)
	Cefmenoxime	NMTT	132.8 ± 2.5†	(74.7)
3	Control		175.9 ± 3.6	(100)
	Latamoxef	NMTT	134.1 ± 4.0*	(76.2)
	Cefmetazole	NMTT	117.1 ± 4.8*	(66.6)
	Cefotiam	DATT	163.9 ± 2.2	(93.2)
	Cefazolin	MTDT	54.2 ± 3.7*	(30.8)
4	Control		157.6 ± 5.0	(100)
	Latamoxef	NMTT	108.1 ± 8.4*	(68.6)
	Cefotetan	NMTT	124.9 ± 5.0*	(79.3)
	Cephalothin	$-CH_2OCOCH_3$	145.0 ± 5.2	(92.0)
5	Control		175.4 ± 3.3	(100)
	Latamoxef	NMTT	105.7 ± 7.8*	(60.3)
	Ceftezole	TDT	27.6 ± 1.8*	(15.7)

The vitamin K-deficient animals were given various antibiotics as shown in Fig. 2 (Exp. 1), at 300 mg/kg, once daily for 3 days, except for experimental group 5 where the dose of antibiotics was 600 mg/kg. Abbreviations of 3′-position substituents of the cephem nucleus: NMTT, *N*-methyltetrazolethiol (1-methyl-1*H*-tetrazole-5-thiol); DATT, 1-(2-dimethylamino)ethyl-1*H*-tetrazole-5-thiol; MTDT, 2-methyl-1,3,4-thiadiazole-5-thiol; TDT, 1,3,4-thiadiazole-5-thiol. Values are means ± SE of four animals, and the number in parentheses is the relative activity.

† Statistically significant ($P < 0.01$ and † $P < 0.05$) against the corresponding control.

Table 4. Effect of vitamin K administration on LMOX-induced changes in blood coagulation parameters and microsomal vitamin K epoxide reductase activity in rats

Exptl group		PT (sec)	APTT (sec)	Prothrombin (units/ml)	PIVKA (units/ml)	Vit. K epoxide reductase (pmol/min/mg protein)
1	Ordinary diet	12.0 ± 0.2	16.9 ± 0.3	175.2 ± 5.5	2.3 ± 0.2	148.1 ± 2.3
	+LMOX	12.0 ± 0.2	17.4 ± 0.3	173.2 ± 7.2	1.9 ± 0.4	111.5 ± 6.5*
2	Vit. K-deficient diet	16.2 ± 1.0	23.9 ± 1.3	70.9 ± 13.2	4.0 ± 0.5	197.3 ± 7.5
	+Vit. K	11.8 ± 0.2†	15.9 ± 0.5*	196.8 ± 4.3*	2.2 ± 0.4†	219.2 ± 6.1
	+LMOX	31.5 ± 4.2	35.7 ± 2.1†	24.6 ± 1.3†	6.4 ± 0.4†	103.7 ± 0.02*
	+LMOX, Vit. K	12.1 ± 0.1†	16.6 ± 0.5*	197.1 ± 5.2*	1.5 ± 0.2†	115.9 ± 8.5*

Animals were treated as shown in Fig. 1 (Exp. 3). Doses of LMOX and vitamin K were 300 mg/kg and 200 μg/kg respectively. Values are means ± SE of four animals.

† Statistically significant ($P < 0.01$ and † $P < 0.05$) against the corresponding control (ordinary or vitamin K-deficient diet alone).

single administration, and the activity was almost normal 5–7 days after the last administration (Fig. 7). These results indicate that the recovery process after suppression of epoxide reductase by multiple administrations of LMOX does not differ from suppression by a single dose.

DISCUSSION

Coumarin anticoagulant drugs antagonize the action of vitamin K and are used extensively to control the level of blood coagulation factors in plasma [1, 2, 20]. Recently, vitamin K-reversible hypoprothrombinemia was observed clinically in patients who had been treated with some β-lactam antibiotics [6–8, 21], as with patients treated with anticoagulant drugs. However, the antibiotic did not produce hypoprothrombinemia in healthy vitamin K-sufficient volunteers [22] or in vitamin K-sufficient rats or dogs [10, 23]. Subsequently, antibiotic-induced hypoprothrombinemia was found in vitamin K-deficient rats, but not in vitamin K-sufficient rats, when the animals were given β-lactam antibiotics containing NMTT, TDT and MTDT at the 3′-position of the cephem nucleus [10, 12, 24–27]. Furthermore, Bechtold *et al.* [9] reported that the antibiotic-induced prolongation of prothrombin time in patients could be normalized by the administration

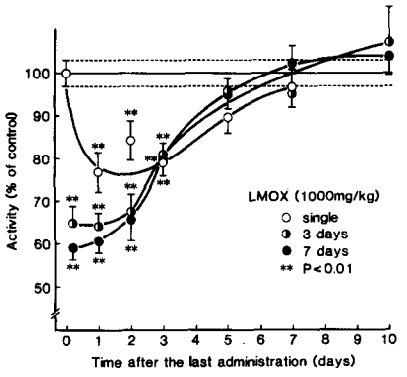

Fig. 7. Time course of the inhibitory effect of LMOX on microsomal vitamin K epoxide reductase. Animals fed the ordinary diet were given a single or multiple administration of LMOX at 1000 mg/kg. Liver samples from rats administered a single dose of LMOX were obtained 1–7 days after the treatment, and the samples from rats injected with LMOX for 3 and 7 days were obtained 4 hr to 10 days after the last administration. Relative activity of microsomal epoxide reductase against the control is plotted as a function of time. Each value is the mean ± SE of four rats. The values (mean ± SE) of the activity in control rats are also shown in the figure as solid and dashed lines. Key: (**) statistically significant ($P < 0.01$) against the corresponding control.

of vitamin K. The efficacy of vitamin K administration for the treatment of antibiotic-induced hypoprothrombinemia was also detected in rats as shown in the present paper (Table 4). The same results have been reported by Oka *et al.* [26] and Matsuura *et al.* [28].

Vitamin K-dependent γ-glutamylcarboxylase catalyzes post-translational modification of the hepatic prothrombin precursor to form biologically activable prothrombin [1, 2]. Lipsky [29, 30] reported the *in vitro* inhibitory action of NMTT-containing antibiotics and NMTT itself on the carboxylase, and proposed that it caused hypoprothrombinemia *in vivo*. However, other investigators could not demonstrate the inhibitory action of these antibiotics in either *in vitro* or *in vivo* systems [10, 24–26, 31–33]. *In vitro* inhibition of γ-carboxylase by NMTT was observed only in the reaction system containing both NMTT and NADH [33, 34], and the inhibitory action of NMTT (*plus* NADH) was suppressed completely by the addition of NADPH [35]. The *in vitro* inhibitory action of NMTT was also reduced by thiol compounds, such as glutathione and cysteine [36]. These results suggest that the *in vitro* inhibition of γ-carboxylase by NMTT is not related to the *in vivo* hypoprothrombinemic action of this compound. In fact, liver microsomes obtained from antibiotic-associated hypoprothrombinemic rats showed normal activities in both γ-glutamylcarboxylase and vitamin K reductase [10, 24–26]. However, the epoxide reductase in these animals was depressed clearly (Tables 2 and 3) as reported previously [11, 12]. The results agree with the clinical report indicating a transient increase in the plasma vitamin K epoxide level when patients treated with antibiotics were given vitamin K [9]. In addition, administration of vitamin K can reverse the abnormalities in blood coagulation parameters even though animals (Table 4) and/or patients [9] are treated with the antibiotics. If antibiotics cause the inhibition of liver γ-carboxylase as proposed previously [29, 30], vitamin K administration should not cause recovery from coagulopathy *in vivo*, because the inhibition of this enzyme seems to cause a decreased capacity for the biosynthesis of coagulation proteins even in the presence of cofactor vitamin K (Fig. 1). Inhibition of the epoxide reductase prevented efficient recycling of vitamin K (Fig. 1) and therefore resulted in an insufficient pool of vitamin K for the carboxylation reaction to occur at a normal rate.

In the present study, we found a close correlation between the depression of microsomal epoxide reductase activity and the development of hypoprothrombinemia during vitamin K-deficiency (Fig. 5 and Table 2). Inhibition of enzyme activity was detected by the administration of antibiotics containing NMTT, TDT and MTDT, and a marked inhibition was observed with TDT- and/or MTDT-containing antibiotics (Table 3). Interestingly, the inhibitory rate caused by NMTT-containing antibiotics was almost constant (20–30%) among the antibiotics examined, suggesting that the heterocyclic thiol compound or NMTT released from the parent antibiotics in the body inhibits the epoxide reductase activity and suppresses efficient recycling of vitamin K. When various NMTT-containing antibiotics are administered intravenously to rats, almost the same amounts of NMTT are liberated in the body and excreted into the urine [37]. Suppression of liver microsomal epoxide reductase activity was observed in rats treated with these heterocyclic thiol compounds (unpublished results). These results support the above assumption. It also suggests that the pharmacokinetic profiles of antibiotics and liberated heterocyclic thiol compounds are important for understanding the developmental mechanism of hypoprothrombinemia, as reported previously [38] concerning the disulfiram-like effect of antibiotics.

NMTT and other heterocyclic thiol compounds inhibited microsomal epoxide reductase activity *in vitro*, although higher concentrations (5–10 mM) were required. Their inhibitory actions were reversed competitively by the addition of increasing concentrations of DTT [19]. However, the decreased microsomal enzyme activity in antibiotic-induced hypoprothrombinemic rats was not restored at all by the addition of higher concentrations of DTT to the assay mixture (data not shown), suggesting that the *in vivo* inhibitory mechanism of these heterocyclic thiol compounds differs from that of the *in vitro* system. Creedon and Suttie [11] and Lipsky [30] suggested that these heterocyclic thiol compounds are required to convert more active metabolites for the exertion of inhibitory action. We cannot presently identify the active metabolite(s) of heterocyclic thiol compounds; this is a subject for further study.

Recovery of vitamin K epoxide reductase activity following its suppression by multiple LMOX administrations was almost the same as that following a single treatment (Fig. 7). The time course of the effect of NMTT-containing antibiotics was similar to that observed for their disulfiram-like effect on the

acetaldehyde-metabolizing enzyme system [37, 39]. It is interesting to note that the time required to normalize the acetaldehyde-metabolizing enzyme (low K_m aldehyde dehydrogenase) [37, 39] and vitamin K epoxide reductase (Fig. 7) was 5–7 days following the administration of antibiotics. These results suggest that the biosynthesis of new enzyme proteins is required for the recovery of the enzyme activities. The results also indicate that patients should be warned to maintain a sufficient vitamin K level for several days after they have been treated with β-lactam antibiotics having NMTT, TDT and MTDT as the 3′-position substituents.

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