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IN VIVO EFFECTS OF β-LACTAM ANTIBIOTICS AND HETEROCYCLIC THIOL COMPOUNDS ON VITAMIN K-DEPENDENT CARBOXYLATION ACTIVITY AND BLOOD COAGULATION FACTORS IN VITAMIN K-DEFICIENT RATS

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Abstract—The *in vivo* effects of heterocyclic thiol compounds, corresponding to the 3′-position substituents of several β-lactam antibiotics, on blood coagulation factors and on liver microsomal γ-glutamylcarboxylation (γ-carboxylation) activity were evaluated in rats maintained on a vitamin K-deficient diet. These rats, when compared to normal control animals, exhibited hypoprothrombinemic changes: prolongation of both prothrombin time and activated partial thromboplastin time, decreases in factor VII and plasma prothrombin, and increases in PIVKA II (descarboxyprothrombin) both in plasma and liver. They also displayed a marked increase in liver microsomal γ-carboxylation activity. These blood coagulation variables could be altered markedly by administering various heterocyclic thiol compounds to the vitamin K-deficient rats, although these compounds did not inhibit γ-carboxylation activity in an assay system using phylloquinone. A similar pattern of alteration was observed when some β-lactam antibiotics were administered. Increased microsomal γ-carboxylation activity in antibiotic-treated vitamin K-deficient rats was normalized by the administration of vitamin K, concomitant with the recovery of blood coagulation variables to the normal range. The results indicate that antibiotic-induced hypoprothrombinemia *in vivo* is not caused by inhibition of enzymes of the γ-carboxylation system, such as vitamin K reductase and γ-glutamylcarboxylase, but is related to the endogenous vitamin K level.

An increased incidence of β-lactam antibiotic-induced hypoprothrombinemia has been proposed to be associated with *N*-methyltetrazolethiol (1-methyl-1*H*-tetrazole-5-thiol, NMTT), a common side chain group at the 3′-position of the cephem or 1-oxacephem frame [1–4]. Lipsky found that NMTT inhibits rat liver microsomal γ-carboxylation activity *in vitro* [5, 6], and he and coworkers suggested that the inhibition would cause hypoprothrombinemia *in vivo* [7]. However, other investigators detected only slight or no inhibition of microsomal γ-carboxylation reaction *in vitro* by NMTT-containing antibiotics or by NMTT itself [8–10]. Subsequent studies have demonstrated that the *in vitro* inhibition of the enzyme activity by NMTT is observable only in the presence of NADH [11, 12, ‡]. In another study§, we examined the *in vitro* inhibitory effects of various five-membered heterocyclic thiol compounds on liver microsomal vitamin K-dependent carboxylation activity under conditions leading to the NMTT action. The enzyme activity was inhibited by all of those compounds, indicating that the *in vitro* action of NMTT is not unique. We thought it of interest to ascertain whether these heterocyclic thiol compounds would produce a hypoprothrombinemic effect. In this paper, we offer evidence that the administration of heterocyclic thiol compounds can lead to hypoprothrombinemia under conditions of vitamin K-deficiency. We also show that vitamin K-dependent carboxylation activity was stimulated in such a hypoprothrombinemic state.

MATERIALS AND METHODS

Animals and their treatments. Sprague–Dawley rats obtained from the Japan Clea Co. Ltd. (Tokyo; Jcl) or from the Shizuoka Laboratory Animals Center (Shizuoka; Slc) were used for the experiments at the age of 8–9 weeks. Animals were kept in an air-conditioned room (25 ± 1°, 50–60% r.h.) lighted 12 hr a day (8:00 a.m. to 8:00 p.m.) and were fed a rat diet (Jcl-CA-1; 500 ng vitamin K/g) and tap water *ad lib*. When vitamin K-deficient rats were used for the experiments, the animals were maintained on a vitamin K-deficient diet and kept in suspended wire-bottomed cages to prevent coprophagy. Two kinds of vitamin K-deficient diet were used for experiments. The diet used exclusively for male rats was prepared in our laboratories, and contained 30–

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‡ K. Uchida and T. Komeno, *Current Advances in Vitamin K Research*, (Ed. J. W. Suttie), p. 477. Elsevier Science Publishers B.V., Amsterdam (1987).

§ T. Oka, A. Touchi, K. Ezumi, M. Yamakawa and T. Matsubara, *Jap. J. Pharmac.* **46**, 165 (1988).

50 ng vitamin K/g. In some experiments, female rats were maintained on a special diet (3–5 ng vitamin K/g) obtained from the TEKLAD Co. (Madison, WI, U.S.A.). β-Lactam antibiotics were dissolved in distilled water and then injected intravenously at 300 or 900 mg/kg body weight. The sodium salts of heterocyclic thiol compounds were dissolved in physiological saline, and the resulting solution was given intravenously at 1 mmol/kg. Phylloquinone was administered to vitamin K-deficient rats subcutaneously at 0.2 mg/kg.

Chemicals. The heterocyclic thiol compounds used in this work were: NMTT, 1,3,4-thiadiazole-5-thiol (TDT), 2-methyl-1,3,4-thiadiazole-5-thiol (MTDT), 1*H*-1,2,3-triazole-5-thiol (2-TT), 1-(2-dimethylamino)ethyl-1*H*-tetrazole-5-thiol (DATT), and 1-hydroxyethyl-1*H*-tetrazole-5-thiol (HTT). All of these compounds and a pentapeptide substrate, Phe-Leu-Glu-Glu-Leu [13], were prepared in the Shionogi Research Laboratories. Radioactive sodium bicarbonate ($NaH^{14}CO_3$; 57.8 mCi/mmol) was obtained from the Amersham Japan Co. Ltd. (Tokyo). Latamoxef (or moxalactam, LMOX) was obtained from Shionogi & Co. (Osaka), cefoperazone (CPZ) from the Toyama Chemical Co. (Tokyo), and cefazolin (CEZ) from the Fujisawa Pharmaceutical Co. (Osaka). Other chemicals of the purest grade available were obtained commercially and used without further purification.

Determination of blood coagulation variables. Venous blood was collected from the vena cava 24 hr after the last drug administration in a 10% vol. of 3.8% sodium citrate under pentobarbital anesthesia, and plasma samples were obtained by centrifugation. Next, liver specimens were removed, and microsomal fractions for the determination of PIVKA II (descarboxyprothrombin) content and γ-carboxylation activity were prepared from the left and median lobes respectively. Prothrombin time (PT), activated partial thromboplastin time (APTT) and factor VII were assayed with COAG-A-MATE-X2 (Warner Lambert), a photo-optical clot detection system based on a one-stage method using fresh plasma. PIVKA II content in plasma and liver and plasma prothrombin were determined as described elsewhere [14].

Determination of γ-carboxylation activity. Vitamin K-dependent γ-carboxylation activity was determined using a microsomal suspension as the enzyme source. The final volume (0.5 ml) of the reaction mixture in SIK buffer (0.25 M sucrose, 50 mM imidazole, 0.5 M potassium chloride, pH 7.2) contained 0.2 ml of microsomal suspension (0.9 to 1.6 mg protein), 0.24% (v/v) Triton X-100, 1 mM pentapeptide substrate, 2 mM NADH, vitamin K cofactor (0.1 mM phylloquinone) and radioactive sodium [^{14}C]bicarbonate solution (10 μCi, 0.35 mM). The reaction was continued in the dark for 60 min at 17° with moderate shaking, and other treatments were carried out as reported previously [12]. Microsomal protein was determined by using a Bio-Rad Protein Assay kit [15] and bovine serum albumin as a standard.

RESULTS

Effects of 3'-side chain analogues of β-lactam antibiotics. When male rats (Jcl) were maintained on a vitamin K-deficient diet for 7 days, hypoprothrombinemic changes in blood coagulation factors, which were not found in rats maintained on ordinary chow, were detected: prolongation of PT, a decrease in plasma prothrombin and an increase in liver PIVKA II (Fig. 1, groups A and B). Under our experimental conditions, the liver microsomal γ-carboxylation activity increased about 3.5 times. Administration of NMTT or TDT to vitamin K-deficient rats promoted each of these changes further (Fig. 1, groups C and D). Two rats, in five, of group D died during the experiment, suggesting a much stronger effect of TDT compared with that of NMTT. Similar patterns in the alterations of blood coagulation factors and

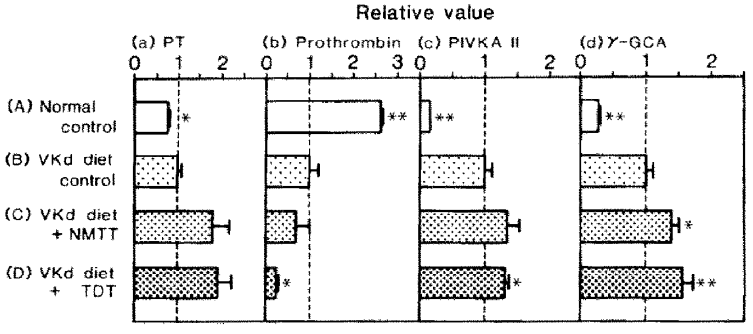

Fig. 1. Effects of NMTT and TDT on blood coagulation. Male rats (Jcl:SD) in group (A) were fed ordinary rat chow and others a vitamin K-deficient diet. The rats in group (B) were given saline (3.6 ml, i.v.) and those in groups (C) and (D) were intravenously administered the test compound (1 mmol/kg, in 3.6 ml saline) once daily for 7 days. The values of (a) prothrombin time, (b) plasma prothrombin, (c) liver PIVKA II and (d) microsomal γ-carboxylation activity (γ-GCA) in groups (A) and (B) were 11.3 ± 0.1 and 14.7 ± 1.1 sec, 169.8 ± 2.6 and 64.5 ± 13.5 NIH thrombin units/ml plasma, 0.11 ± 0.00 and 0.64 ± 0.06 units/mg of liver microsomal protein, and 939 ± 49 and 3310 ± 330 dpm/mg protein/hr respectively. The relative values against the vitamin K-deficient control group (broken line) are represented in the figure. Each bar in the figure indicates the mean ± SE of five animals, except in the case of group (D), where the data came from three rats. Key: (*) and (**) statistically significant ($P < 0.05$ and $P < 0.01$ respectively) against group (B).

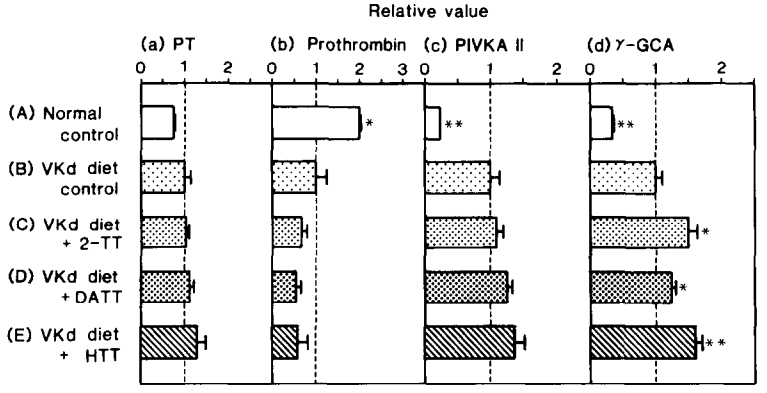

Fig. 2. Effects of 3′-side chain analoges of β-lactam antibiotics on coagulation factors. Male rats (Jcl:SD) in group (A) were fed ordinary rat chow, and other animals were given a vitamin K-deficient diet. Rats in group (B) were given saline (3.6 ml, i.v.) and those in groups (C)–(E) were intravenously administered test compounds (1 mmol/kg, in 3.6 ml saline) once daily for 7 days. All values represent the mean ± SE of five animals. Other conditions were similar to those given in the legend of Fig. 1.

liver microsomal γ-carboxylation activity were observed when 2-TT, DATT or HTT was administered (Fig. 2). When animals were given MTDT, four rats, in five, of the group died during the experiment and, thus, the data were removed from the figure. The activity of liver microsomal γ-carboxylation was increased approximately 50% by these compounds.

Effects of antibiotics and vitamin K. As described above (Figs. 1 and 2), various heterocyclic thiol compounds, which are the analogues of the 3′-position substituent of β-lactam antibiotics, caused both an increase in liver PIVKA II and a stimulation in liver γ-carboxylase activity, although the *in vivo* enzyme reaction decreased, presumably, under the vitamin K-deficient conditions. Next, the effects of several antibiotics on blood coagulation variables and on liver microsomal γ-carboxylation activity of hypoprothrombinemic animals were compared with those of heterocyclic thiol compound-treated rats. As shown in Fig. 3, administration of CEZ, CPZ or LMOX to the vitamin K-deficient rats resulted in prolongation of PT and a decrease in plasma prothrombin. Under the experimental conditions employed, vitamin K deficiency-induced increases in PIVKA II content and γ-carboxylation activity in liver were not stimulated further by the administration of antibiotics. The results indicated that the alterations of blood coagulation factors and liver γ-carboxylase activity caused by thiol compound administration were detected in the antibiotic-treated animals in essentially the same manner.

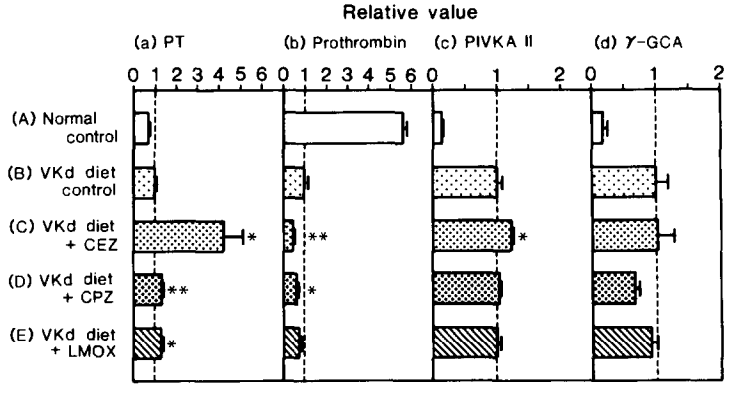

Fig. 3. Effects of various β-lactam antibiotics on coagulation factors and γ-carboxylation activity. Female rats (Slc:SD) in group (A) were fed an ordinary diet and others were fed the TEKLAD vitamin K-deficient diet for 10 days. The vitamin K-deficient rats were further treated with intravenous administration of antibiotics at 900 mg/kg (in 3.6 ml saline) once daily throughout the experiment. The values of (a) prothrombin time, (b) plasma prothrombin, (c) liver PIVKA II, and (d) microsomal γ-carboxylation activity in groups (A) and (B) were 11.0 ± 0.2 and 16.2 ± 1.1 sec, 240.2 ± 7.8 and 42.8 ± 5.5 NIH thrombin units/ml plasma, 0.12 ± 0.01 and 0.84 ± 0.07 units/mg of liver microsomal protein, and 660 ± 216 and 3490 ± 624 dpm/mg protein/hr respectively. The relative values against the vitamin K-deficient control (broken line) are represented in the figure. Each bar in the figure indicates the mean ± SE of five animals. Key: (*) and (**) statistically significant ($P < 0.05$ and $P < 0.01$ respectively) against group (B).

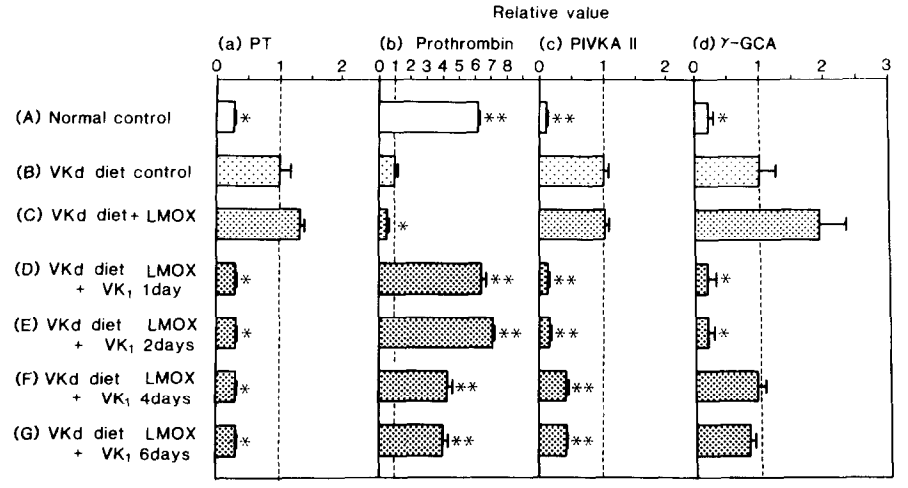

Fig. 4. Effects of vitamin K_1 supplement on coagulation factors and γ-carboxylation activity of vitamin K-deficient rats with antibiotic treatment. Male rats (Slc) in group (A) were fed an ordinary diet and groups (B)–(G) were fed TEKLAD vitamin K-deficient diet for 4 days. Next, the animals were given a single subcutaneous administration of phylloquinone (0.2 mg/kg) and maintained vitamin K-deficient for 1–6 days. The vitamin K-deficient rats were intravenously given LMOX (300 mg/kg, in 3.6 ml saline) once daily throughout the experiment. The values of (a) prothrombin time, (b) plasma prothrombin, (c) liver PIVKA II, and (d) liver microsomal γ-carboxylation activity in groups (A) and (B) were: 11.0 ± 0.1 and 38.3 ± 6.2 sec, 187.9 ± 3.8 and 30.2 ± 5.9 NIH thrombin units/ml plasma, 0.10 ± 0.01 and 0.90 ± 0.08 units/mg of liver microsomal protein, and 347 ± 125 and 1570 ± 380 dpm/mg protein/hr respectively. The relative values against the vitamin K-deficient control (broken line) are as given in the legend of Fig. 3.

After the development of hypoprothrombinemic change in coagulation factors in vitamin K-deficient rats administered the NMTT-containing antibiotic LMOX, the animals were given a single subcutaneous dose of phylloquinone and were further maintained for 1–6 days on the vitamin K-deficient diet with daily administration of LMOX. Next, the blood coagulation factors and liver γ-carboxylation activity were determined. Figure 4 shows that hypoprothombinemic changes in PT, plasma prothrombin and liver PIVKA II content recovered to the normal range 1 and 2 days after the administration of vitamin K, even in the LMOX-treated vitamin K-deficient rats. Both plasma PIVKA II and factor VII levels or PT and APTT levels showed similar patterns of recovery (data not shown). The blood coagulation factors changed gradually to the hypoprothrombinemic values 4–6 days after the vitamin K treatment. Liver γ-carboxylation activity showed a pattern parallel to that of the liver PIVKA II level; both increased under the hypoprothrombinemic states and rapidly decreased to the normal level after vitamin K treatment. The results shown in Fig. 4 indicate clearly that both liver γ-glutamylcarboxylase and vitamin K reductase were not inhibited in the antibiotic-induced hypoprothrombinemic state with vitamin K-deficiency, and the supplement of vitamin K led to the recovery of all the blood coagulation variables to normal levels.

DISCUSSION

Administration of the antibiotic LMOX or its side chain, NMTT, prolongs PT in vitamin K-deficient rats [7] but does not produce hypoprothrombinemia in vitamin K-sufficient rats, dogs or human volunteers [16, 17]. Furthermore, vitamin K administration reverses antibiotic-induced hypoprothrombinemia in patients [18]. These findings suggest that the vitamin K level in the body is an important factor in the development of hypoprothrombinemia, and microsomal γ-carboxylase is essentially not inhibited by β-lactam antibiotics or by their fragments, *in vivo*.

As reported by several investigators [1–3, 18] for their patients, vitamin K deficiency also causes hypoprothrombinemic changes in blood coagulation factors in rats [7, 14], that is, prolongation of PT and APTT, decreases in plasma prothrombin and factor VII, and increase in PIVKA II both in liver and plasma [14]. Our data agreed well with these results. Administration of antibiotics (Fig. 3) or their fragmental analogues, heterocyclic thiol compounds (Figs. 1 and 2), resulted in the enhancement of hypoprothrombinemic changes. Sex-related differences in the development of hypoprothrombinemia are well recognized in rats, with male rats responding more severely to vitamin K deficiency than female rats [14, 19–21]. When animals were maintained on a vitamin K-deficient diet containing 30–50 ng vitamin K/g, the hypoprothrombinemic changes in blood coagulation variables were detected only in male rats and not in female rats. The changes due to vitamin K deficiency were detected in female rats after feeding of the special diet containing 3–5 ng vitamin K/g. We employed both male and female rats for the experiments. Except for the vitamin K level in the diet, there were no essential differences between the two sexes in the hypoprothrombinemic

states caused by the vitamin K deficiency or in the effects of the antibiotics.

Interestingly, liver microsomal enzymes obtained from hypoprothrombinemic rats showed much higher vitamin K-dependent γ-carboxylation activity than those from normal animals (Figs. 1–4), and the activity seemed to correlate negatively with the level of prothrombin and/or with that of factor VII (data not shown). Administration of vitamin K returned all of the blood coagulation factors and γ-carboxylation activity to their normal levels, within 1 day at the latest (Fig. 4). We concluded from these results that drug administration advances hypoprothrombinemia of vitamin K-deficient rats, probably due to enhancement of the vitamin K deficiency. This causes a decrease in the γ-carboxylation reaction *in vivo*, and then leads to accumulation of PIVKA II in the body. As a matter of fact, administration of the drug tends to increase the activity of liver microsomal γ-carboxylase but the vitamin K-deficiency seems to restrict the over-all efficiency of the γ-carboxylation reaction. Thus, a supplement of vitamin K led to recovery of all of these abnormalities into the normal range. This agrees well with observations that antibiotic-induced hypoprothrombinemia in humans [1–3, 18] or rats (Fig. 4) was reversed quickly by vitamin K administration.

Various β-lactam antibiotics and their fragmental compounds inhibit microsomal γ-carboxylation activity directly under certain special conditions *in vitro* [8, 12, *]. The administration of these compounds further enhances hypoprothrombinemia of vitamin K-deficient rats [7]. However, the activity of γ-carboxylase is not inhibited but rather activated by this treatment. These observations suggest that *in vivo* enhancement of hypoprothrombinemia by drug administration is related not with *in vitro* inhibition of γ-carboxylase, but rather with endogenous vitamin K levels. Acute administration of phylloquinone is followed by the transient appearance of vitamin K-2,3-epoxide in patients who showed antibiotic-related hypoprothrombinemia [18], suggesting a depression of liver microsomal vitamin K epoxide reductase activity. In fact, administration of NMTT causes a marked decrease in liver vitamin K-epoxide reductase activity in rats [22], and we obtained the same results using antibiotic-treated rats (unpublished results). All these results support the above assumption. This impaired hepatic regeneration of vitamin K was considered to decrease endogenous vitamin K level [18, 22], and the recovery from antibiotic-related hypoprothrombinemia by vitamin K administration supports this consideration.

These situations resemble very closely those postulated for the coumarin-like effect of NMTT-containing antibiotics [18] and of NMTT [22]. Therefore, as has been documented for coumarin anticoagulants [23–25], various β-lactam antibiotics and their fragmental compounds seem to inhibit vitamin K-2,3-epoxide reductase.

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