Vitamin K reversible hypoprothrombinemia in rats. II. Efficacy of vitamin K on latamoxef-induced coagulopathy in rats.

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Accepted April 21, 1988

Abstract—Feeding of a vitamin K-deficient diet caused the development of hypoprothrombinemic changes in rats such as prolongation of prothrombin time (PT) and activated partial thromboplastin time (APTT), decreases in plasma prothrombin and clotting factor VII levels, and an increase in the descarboxyprothrombin (PIVKA) level in both plasma and liver. Successive administrations of latamoxef (LMOX) to the vitamin K-deficient rats resulted in the further enhancement of these changes. After the development of hypoprothrombinemia with LMOX, a single subcutaneous injection of vitamin K normalized most of these abnormalities in blood coagulation parameters within 6 hr. When vitamin K was given at 200 μg/kg, PT, APTT and the plasma PIVKA level showed normal values for at least 8 days even when the animals were fed a vitamin K-deficient diet and treated with LMOX during the recovery period. The amount of vitamin K required to maintain most of the blood coagulation parameters in the normal range was about 3 μg/kg/day. The plasma level of vitamin K was higher than 0.3–0.5 ng/ml when the blood coagulation parameters were maintained in the normal range.

Several beta-lactam antibiotics, possessing an N-methyl-tetrazolethiomethyl group at the 3-position of the cephem nucleus, have been reported to cause hypoprothrombinemia in vitamin K-deficient humans (1–8) and experimental animals (9–11). Our previous study (12) also revealed that some beta-lactam antibiotics containing N-methyl-tetrazolethiol (1-methyl-1H-tetrazole-5-thiol, NMTT), thiadiazolethiol or methyl-thiadiazolethiol produced hypoprothrombinemia in rats fed a vitamin K-deficient diet. However, these antibiotics caused no hypoprothrombinemic changes in blood coagulation parameters when the rats were maintained on an ordinary diet containing a sufficient amount of vitamin K (9).

Three enzymes preferentially involved in vitamin K metabolism and coagulation factor synthesis (Fig. 1). Gamma-glutamylcarboxylase plays a fundamental role in the synthesis of vitamin K-dependent coagulation factors. Vitamin K reductase is also important for the formation of a cofactor, the hydroquinone form of vitamin K, for gamma-glutamylcarboxylase, since vitamin K is supplied in an ineffective quinone form. Vitamin K epoxide reductase is also required to complete the vitamin K cycle to generate the quinone form of vitamin K (13). Several investigators have revealed that the administration of NMTT-containing antibiotics and NMTT itself caused the depression of vitamin K epoxide reductase (14, 15), but not of gamma-glutamylcarboxylase and vitamin K reductase (9, 10, 16). These results agree with the above data that the NMTT-containing antibiotics caused no hypoprothrombinemic changes in the presence of sufficient amounts of vitamin K.

We thought it of interest to check whether...
the antibiotics-induced hypoprothrombinemic changes in blood coagulation parameters can be recovered by the administration of vitamin K. Here we describe the recovery process of blood coagulation parameters after the administration of vitamin K to the latamoxef (LMOX) induced hypoprothrombinemic rats and also report the amount of vitamin K required to maintain these parameters in the normal range.

Materials and Methods

Animals and their treatments: Slc-Sprague Dawley strain female rats, aged 7 weeks, were used for the experiments. The animals were kept in an air-conditioned room (23±2°C, 55±10% relative humidity) lighted 12 hr a day (8:00-20:00). Rats were fed an ordinary diet (CA-1, Clea Japan, Inc., Tokyo) or a vitamin K-deficient diet (Oriental Yeast Co., Tokyo or TD81053, Teklad Co., Madison, WI, U.S.A.). The vitamin K contents in the ordinary and the vitamin K-deficient diet were about 500 ng/g and less than 5 ng/g, respectively. During the experiments, the rats were housed in wire-bottomed cages (2 rats per cage) to prevent vitamin K intake by co-propagation.

Hypoprothrombinemic states in rats were induced by maintaining them on the vitamin K-deficient diet for 10 days or by the intravenous administration of LMOX at 900 mg/kg/day, once daily for 10 days, under the vitamin K-deficiency. Then, a single subcutaneous administration of vitamin K was performed, and blood and liver samples were obtained periodically for the determination of blood coagulation parameters and vitamin K concentrations. Feeding of the vitamin K-deficient and LMOX treatment were continuously carried out after the vitamin K treatment. Blood samples were obtained from the vena with a disposable syringe containing 1/10 volume of 3.8% sodium citrate solution under ether anesthesia.

Antibiotics and vitamin K: LMOX obtained from Shionogi & Co. (Osaka) was dissolved in distilled water at the concentration of 25% (w/v), and the resulting solution was injected intravenously at the speed of 2 ml/min. Animals in the control group were given physiological saline intravenously at 3.6 ml/kg. Vitamin K (vitamin K1, Eisai Co., Tokyo) was diluted with distilled water and administered subcutaneously.

Determination of blood coagulation parameters: Prothrombin time (PT), activated partial thromboplastin time (APTT) and clotting factor VII were measured with a photooptical clot sensing system, COAG-A-Mate-X2 (Warner Lambert Co., Morris Plains, NJ, U.S.A.). Plasma levels of PIVKA (protein induced by vitamin K absence, descarboxyprothrombin) and prothrombin were determined as described previously (17).

Determination of vitamin K: The plasma concentration of vitamin K was determined by a high performance liquid chromatographic method as reported by Hirauchi et al. (18). The liver content of vitamin K was measured as described previously (19).

Results

Reversibility of antibiotic-induced coagulopathy by vitamin K: As described previously (12), feeding of a vitamin K-deficient diet caused hypoprothrombinemic changes in rats: prolongation in PT and APTT, decreases in factor VII and prothrombin levels, and increase in plasma PIVKA level (Fig. 2, open
circles). Administration of LMOX, one of the NMTT-containing antibiotics, enhanced these changes in the blood coagulation parameters (Fig. 2, closed circles). After the development of the hypoprothrombinemic changes in the coagulation parameters by long-time (10 days) feeding of vitamin K-deficient diet or repeated administrations of LMOX under vitamin K-deficient states as described in "Methods", vitamin K was given subcutaneously, and its effect on blood coagulation parameters was determined periodically. The APTT and plasma factor VII level normalized quickly within 6 hr after the vitamin K treatment regardless of LMOX administration (Fig. 2, A and B). PT and liver PIVKA content also showed a recovery as rapid as that of APTT data not shown). On the other hand, plasma prothrombin and PIVKA levels in both groups of rats normalized slowly, attaining the normal range 24–48 hr after the vitamin K treatment (Fig. 2, C and D).

Plasma and liver vitamin K levels in normal rats fed the ordinary diet were 2.39±6.7 ng/ml (n=6) and 29.16–30.35 ng/g (n=2), respectively, and the levels decreased to 0.17±0.08 ng/ml (n=6) and 1.37–1.73 ng/g (n=2) ("0" time in Fig. 3), respectively, by the long-time feeding of the vitamin K-deficient diet. When 200 μg/kg vitamin K₁ was injected into rats on the deficient diet, very high vitamin K₁ levels were detected in the plasma and liver after 6 and 12 hr, and then the levels decreased gradually (Fig. 3). The results shown in Figs. 2 and 3 indicate clearly that the coagulopathy induced by vitamin K deficiency and/or antibiotics treatment can be normalized easily and quickly by the administration of vitamin K, concomitant with the increase in plasma and liver vitamin K level.

**Determination of the duration of vitamin K action:** After a single injection of vitamin K
at 200 µg/kg to vitamin K-deficient rats or LMOX-treated rats under vitamin K deficiency, blood samples were collected 2, 4, 6 and 8 days later. The APTT and plasma PIVKA level were normalized by the vitamin K injection for at least 8 days in both groups of rats (Fig. 4, A and D). Normalized PT values were also detected during the 8-day recovery period (data not shown). A single vitamin K injection resulted in normalization of the plasma factor VII and prothrombin levels over 6–8 days in the vitamin K-deficient rats (Fig. 4, B and C, open circles). On the other hand, the factor VII and prothrombin levels in vitamin K-deficient LMOX-treated rats normalized in only 1–2 days after the vitamin K administration, and thereafter gradually shifted to hypoprothrombinemic levels (Fig. 4, B and C, closed circles). Plasma vitamin K levels of 0.25–0.71 ng/ml were detected 2–8 days after the vitamin injection. These results...

Fig. 3. Alterations of vitamin K level in plasma and liver following administration of vitamin K. Plasma and liver samples were obtained from the animals used for the Fig. 2 experiments. The plasma vitamin K level in the figure represents the mean and standard error of 6 rats, while the liver content is the mean value of 2 rats.

Fig. 4. Effect of vitamin K administration on blood coagulation parameters in LMOX-treated rats. Vitamin K-deficient animals were subjected to LMOX treatment (900 mg/kg/day) for 10 days (closed circles) or no treatment (open circles), and then they received a single administration of vitamin K at 200 µg/kg. Thereafter, feeding of the deficient diet and the LMOX treatment were continued, and blood samples were obtained 2–8 days after the vitamin K treatment. Values of the blood coagulation parameters in the ordinary diet-fed normal rats are shown in the figure by the dashed line. The values of APTT (A), clotting factor VII (B), prothrombin level (C) and PIVKA level (D) are shown in the figure as the mean and standard error of 6 animals. * and **, statistically different (P<0.05 and P<0.01, respectively) from the control ("0" time).
demonstrate that the administration of 200 µg/kg of vitamin K causes normalization of all blood coagulation parameters for about 4–6 days even when the antibiotic treatment is continued.

Effects of various doses of vitamin K on blood coagulation parameters: Vitamin K-deficient animals were treated with LMOX for 10 days and then given a single subcutaneous injection of vitamin K at various doses (0.3–200 µg/kg). Blood samples were obtained 1 and 4 days after the vitamin administration.

Vitamin K deficiency dependent hypoprophthalmicemic changes in blood coagulation parameters (Fig. 5, group b) were further enhanced by the administration of LMOX as shown in the figure (group c). Administration of a 0.3–1.0 µg/kg dose of vitamin K partially normalized the blood coagulation parameters in the LMOX-treated rats 1 day after the vitamin K treatment, and almost complete recoveries of all these parameters were detected in rats administered a 3.0 or 200 µg/kg dose of vitamin K (Fig. 5, groups d to g).

Fig. 5. Effect of various doses of vitamin K on blood coagulation parameters. Animals fed the vitamin K deficient diet were treated with LMOX at 900 mg/kg, once daily for 10 days, and then they received a single injection of various doses of vitamin K. The animals were further maintained on the deficient diet with daily administration of LMOX, and their blood samples were collected 1 and 4 days after the vitamin K injection. Animals in group (a) and groups (b)–(g) were maintained on the ordinary and vitamin K-deficient diet, respectively. LMOX was injected into the rats in groups (c)–(g). Animals in groups (d)–(g) were given a single subcutaneous injection of vitamin K at doses of (d) 0.3, (e) 1.0, (f) 3.0 and (g) 200 µg/kg. The values in the figure represent the mean and standard error of 5–6 rats. * and **, statistically significant (P<0.05 and P<0.01, respectively) against the corresponding LMOX-treated vitamin K-deficient rats (group c).
The normalization of blood coagulation parameters in LMOX-treated rats was observed even after 4 days when animals were given a 200 μg/kg dose of vitamin K, but not with lower doses. This shows that the minimum dose of vitamin K required to maintain blood coagulation parameters in the normal range is about 3 μg/kg/day, and higher doses can normalize the parameters for longer periods.

The plasma level of vitamin K was determined 1 and 4 days after the administration of various doses of vitamin K. The level increased dose-dependently 1 day after the vitamin K administration, and an extremely high plasma level of vitamin K was detected when a 200 μg/kg dose of the vitamin was given (Table 1). However, the plasma concentration of vitamin K decreased 4 days after the administration. When the blood coagulation parameters in LMOX-treated rats were normalized (Fig. 5), the plasma vitamin K level was 0.3–0.7 ng/ml (Table 1).

**Correlation between plasma vitamin K level and blood coagulation parameters:** As

| Exptl group | Diet         | Administration | Plasma vitamin K (ng/ml) |
|-------------|--------------|----------------|-------------------------|
|             | LMOX (mg/kg) | Vitamin K (μg/kg) | 1 day after vitamin K | 4 days after vitamin K |
| a           | Ordinary     | 0              | 0.21 ± 0.03 (6)         | 2.34 ± 0.34 (6)        |
| b           | K-Deficient  | 0              | 0.18 ± 0.09 (4)         | 0.13 ± 0.04 (6)        |
| c           | K-Deficient  | 900            | 0.3                      | 0.16 ± 0.06 (5)        |
| d           | K-Deficient  | 900            | 1.0                      | 0.11 ± 0.01 (3)        |
| e           | K-Deficient  | 900            | 3.0                      | 0.38 ± 0.04 (6)        |
| f           | K-Deficient  | 900            | 45.01 ± 6.84 (5)         | 0.14 ± 0.02 (6)        |
| g           | K-Deficient  | 900            | 200.0                    | 0.73 ± 0.10 (6)        |

Animals were maintained on the ordinary or vitamin K-deficient (K-Deficient) diet, and they were treated with LMOX and vitamin K as described in Fig. 5. Dosages of LMOX and vitamin K are shown in the table. Plasma samples were obtained 1 day and 4 days after the vitamin K administration, and plasma levels of vitamin K were detected by HPLC method. The values in the table represent the mean ± S.E., and the number in parenthesis is the number of animals used for the determination.

**Fig. 6.** Relationships between plasma vitamin K level and prothrombin time (A) or plasma prothrombin level (B). The blood coagulation parameters determined 1 day after the vitamin K administration are plotted as a function of the plasma vitamin K level. The values of the 200 μg/kg dose-group were omitted from the figure.
mentioned above, LMOX-induced hypoprothrombinemic changes in coagulation parameters were reversed by vitamin K injection. We checked the individual relationships between plasma vitamin K level and the coagulation parameters in order to find the minimum plasma vitamin K level needed to maintain the normal prothrombin time. Using the data shown in Fig. 5, individual PT and plasma prothrombin levels were plotted against the plasma vitamin K level (Fig. 6). When the plasma vitamin K level was lower than 0.3–0.5 ng/ml, prolongation of PT was detected regardless of the treatment, while the rats with higher levels of vitamin K (higher than 0.3–0.5 ng/ml) showed normal PT values (Fig. 6A). A similar pattern of correlation was obtained when APTT and PIVKA levels in plasma and liver were plotted against the vitamin K level (data not shown). On the other hand, the plasma prothrombin level showed higher values when the vitamin K concentration was higher than 0.5 ng/ml, and the prothrombin level decreased gradually concomitant with the decrease in vitamin K concentration (Fig. 6B). Similar patterns were obtained when the factor VII levels in plasma were plotted against the vitamin K concentration in plasma (data not shown). These results, shown in Fig. 6, indicate that the minimum plasma concentration of vitamin K for maintaining blood coagulation parameters in the normal range is 0.3–0.5 ng/ml.

Discussion

Beta-lactam antibiotics, having NMTT as the 3'-position substituent, have been reported to cause hypoprothrombinemia in patients (1–8), but LMOX, one of these antibiotics, and NMTT itself do not produce abnormalities in blood coagulation parameters in human volunteers or in vitamin K-sufficient animals (9, 20, 21). Furthermore, administration of vitamin K reversed antibiotic-induced hypoprothrombinemia in patients (22). All these findings suggest that the vitamin K level in the body is an important factor in the appearance of hypoprothrombinemia. We also demonstrate in this paper that the LMOX-induced hypoprothrombinemia in vitamin K-deficient female rats can be easily and quickly reversed by the administration of vitamin K. Interestingly, a high dose of vitamin K (200 μg/kg) normalized the blood coagulation parameters in rats for about 4–6 days even though the animals were continuously treated with the antibiotic (Figs. 2 and 4).

Lipsky reported the in vitro inhibition of the liver microsomal gamma-glutamylcarboxylation reaction by NMTT and proposed that it causes hypoprothrombinemia in vivo (23). If antibiotics did cause the inhibition of liver carboxylase as he proposed, 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 blood coagulation proteins even in the presence of sufficient amounts of cofactor vitamin K (Fig. 1). However, we found that administration of vitamin K to the antibiotic-treated rats normalized the abnormalities in PT, APTT, and plasma levels of factor VII, prothrombin and PIVKA, as in the animals with vitamin K deficiency (Fig. 2). When vitamin K is administered, it is supplied as the quinone form and must be converted to the hydroquinone form by vitamin K reductase as depicted in Fig. 1. The biosynthetic reaction of gamma-glutamylcarboxylase proceeds in the presence of cofactor, the hydroquinone form of vitamin K (13). Thus, the results shown in this paper indicate no inhibition of both gamma-glutamylcarboxylase and vitamin K reductase (Fig. 1), although the in vivo functions of these enzymes were suppressed under the vitamin K deficiency. Several investigators have demonstrated no inhibition of microsomal gamma-glutamylcarboxylase by NMTT-containing antibiotics and NMTT itself (9, 10, 16, 24, 25), which supports the above assumption.

Clinical studies by Bechtold et al. demonstrated an increase in the plasma concentration of vitamin K epoxide in antibiotics-pretreated patients following the administration of vitamin K (22), which suggested the inhibition of vitamin K epoxide reductase by these antibiotics. Subsequent studies revealed that the NMTT-containing antibiotics and NMTT itself cause the inhibition of microsomal vitamin K epoxide reductase in rats (14, 15). These clinical and experimental findings
indicate impairment of the hepatic vitamin K metabolism, which is followed by hypoprothrombinemia under vitamin K-deficient conditions. Administration of vitamin K reversed the antibiotics-induced hypoprothrombinemia in both patients (22) and experimental animals as shown in this paper. When a 200 μg/kg dose of vitamin K was injected, extremely high concentrations of the vitamin were detected in the plasma and liver 6-12 hr after the injection, and thereafter gradually disappeared (Fig. 3). The disappearance profiles of the vitamin were almost the same in both plasma and liver, suggesting that alteration of the plasma vitamin K level reflects a variation in the vitamin K content of the liver. We also examined the relationship between the plasma concentration of vitamin K and the blood coagulation parameters (Fig. 6), which might demonstrate the amount of hepatic vitamin K required for the biosynthesis of clotting factors. Our data indicated that 0.3–0.5 ng/ml is the minimum plasma concentration of vitamin K needed to keep the blood coagulation parameters in the normal range (Fig. 6), and daily administration was required to maintain the minimum vitamin K level of 3 μg/kg/day (Fig. 5 and Table 1). These results suggest that the clinical application of vitamin K at 10 mg/man (26) supplies enough for the biosynthesis of clotting factors.

In conclusion, the hypoprothrombinemic changes in blood coagulation parameters can be easily detected in rats fed a vitamin K-deficient diet. Administration of LMOX, one of the NMTT-containing antibiotics, causes the enhancement of vitamin K-deficiency concomitant with the development of coagulopathy. However, this can be quickly reversed by the administration of vitamin K. When vitamin K is injected subcutaneously, the minimum amount required to maintain blood coagulation parameters in the normal range is 3 μg/kg/day.

Acknowledgments: We thank Dr. T. Yoshizaki and Messrs. T. Takano and T. Harauchi for determining the blood coagulation parameters. We thank also Dr. K. Hirauchi and Mr. T. Sakano for their assistance in vitamin K determination.

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