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COMPARISON OF INTESTINAL ABSORPTION OF VITAMIN K_2 (MENAQUINONE) HOMOLOGUES AND THEIR EFFECTS ON BLOOD COAGULATION IN RATS WITH HYPOPROTHROMBINAEMIA

YASUHIRO AKIYAMA,* KUNIKO HARA, AKIRA MATSUMOTO, SUMIKO TAKAHASHI and TETSUYA TAJIMA

Department of Drug Research II, Eisai Co. Ltd, 4-6-10, Koishikawa, Bunkyo-ku, Tokyo 112-88, Japan

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Abstract—To examine the physiological activities of vitamin K_2 (menaquinone, MK) homologues with different numbers of isoprene units, MK with 1–14 isoprene units and menadione (MK-0) were administered to rats with hypoprothrombinaemia, and the absorption, concentration in liver and ameliorating effect of these MK on hypoprothrombinaemia were compared. Hypoprothrombinaemia was induced by giving a vitamin K-deficient diet and warfarin (0.06 mg/kg body weight) for 8 days. Before MK treatment, the MK were undetectable in plasma and liver. At 6 hr after oral MK administration (0.1 mg/kg): MK was not detected in the plasma in rats treated with MK with 1, 2, 3 or more than 12 isoprene units; the MK level in the liver was increased but blood coagulation activity was not improved in rats treated with MK with 0, 9, 10 or 11 isoprene units; the MK level in the liver was increased and hypoprothrombinaemia was slightly improved in rats treated with MK with 7 or 8 isoprene units; and the MK level in the liver was increased and hypoprothrombinaemia was markedly improved in rats treated with MK with 4, 5 or 6 isoprene units. Almost identical results were observed 3 hr after intravenous injection of MK with 4, 5 or 6 isoprene units (10 nmol/kg). These findings suggest that the number of isoprene units of MK is an important factor in its absorption and incorporation into the liver and that the ameliorating effect of MK on hypoprothrombinaemia does not parallel their concentrations in the liver.

Key words: menaquinone; blood coagulation activity; absorption; MK concentration in liver; rats

Vitamin K is an essential cofactor for the production of clotting factors II, VII, IX, and X in mammals [1, 2]. Vitamin K exists in two major forms: K_1 (phylloquinone) and K_2 (MK†). MK, as shown in Fig. 1, has 14 homologues according to the number of isoprene units at the 3 position of the quinone structure. Vitamin K_1 is found primarily in green leafy vegetables [3] while MK is of microbial origin: they are found mainly in fermented foods [4] as well as the colon, where they are produced by the intestinal flora [5, 6]. The human vitamin K requirement is assumed to be satisfied mainly by dietary phylloquinone and menaquinones with short isoprene units, but controversy exists as to whether bacteria-produced MK with longer isoprene units contributes to meet the vitamin K requirement of the host. Some reports suggest that bacteria-produced MK is utilized in the maintenance of blood coagulation, as starvation alone could not induce vitamin K deficiency while starvation combined with antibiotic therapy sometimes caused hypoprothrombinaemia in humans [7, 8]. While several MK with long isoprene units are reported to be present in human liver [9], they contribute little to clotting factor synthesis [10].

Only a few reports [11, 12] have compared the effects of MK with various numbers of isoprene units on hypoprothrombinaemia, and the conclusions of these reports are not consistent. No report has yet simultaneously compared absorption from the intestine and the physiological activity of MK homologues. Therefore, menadione and MK (1 to 14 isoprene units) were administered to rats with hypoprothrombinaemia and we observed their plasma concentrations, incorporation into the liver and ameliorating effects.

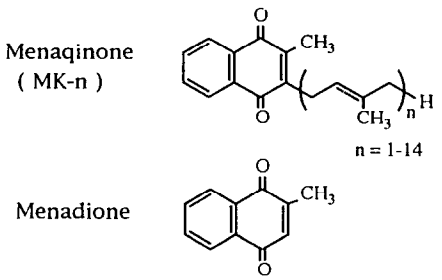

Fig. 1. Chemical structure of MK-*n* and menadione.

MATERIALS AND METHODS

Animals and diets. Male Sprague–Dawley rats

* Corresponding author. Tel. 813-3817-5279; FAX 813-3811-6830.

† Abbreviation: MK, menaquinone.

Table 1. Composition (%) of vitamin K-deficient diet

Protein	
Vitamin-free casein	24.5
Carbohydrates	
Corn starch	45.5
Granulated sugar	10.0
α-Corn starch	1.0
Fat	
Palm oil	6.0
Fibres	
Abicel cellulose	3.0
KC-frock	2.0
Vitamin mix (without vitamin K)	1.0
Mineral mix	7.0

(6-weeks-old, Charles River Co., Tokyo, Japan) were housed in stainless-steel, wire bottomed cages. They were kept in an air conditioned room at 24° with a 12 hr light, 12 hr dark cycle.

The rats were allowed free access to a conventional diet (CE-2, Japan Clea Inc., Tokyo, Japan) and distilled water for 1 week. They were then divided into two groups: normal and vitamin K-deficient groups. Rats in the normal group were fed a conventional diet and distilled water. Rats in the vitamin K-deficient group were fed a vitamin K-deficient diet (Japan Clea Inc.). The composition of the diet is listed in Table 1. The total vitamin K (K_1 and K_2) content was less than 5 ng/g diet. These rats were also given potassium warfarin (Warfarin®, Eisai Co. Ltd, Tokyo, Japan) in drinking water (0.39 mg of warfarin per litre of water) for 8 days. The mean daily dose of warfarin was 0.06 mg/kg body weight, calculated from the mean body weight and water intake. In the oral administration experiment, MK was given to rats under non-fasting conditions.

Materials. Fourteen homologues of vitamin K_2 (MK-*n*, *n*: the number of isoprene units at the 3-position of quinone structure, $n = 1$–14) and menadione (MK-0) which have no isoprene units were examined. MK-0 was obtained from Tokyo Kasei Kogyo Co. Ltd (Tokyo, Japan). MK with 1–14 isoprene units were synthesized by Dr K Shimada *et al.* in Eisai Co. Ltd. MK with more than 2 isoprene units were identified to be in all *trans* configuration. Figure 1 shows the chemical structure of MK-n. The amounts of MK homologues in food and intestinal contents are reported on a weight basis. Therefore, vitamin K_2 homologues were orally administered on a weight basis in experiments 1 and 2. As their common chemical structure, the naphthoquinone component, contributes to physiological activities of vitamin K, it was also important to compare their physiological activity on a molar basis. Therefore, in experiment 3, the same doses of MK on a molar basis were intravenously administered.

Experiment 1. Hypoprothrombinaemic rats induced by a vitamin K-deficient diet and warfarin for 8 days were divided into 33 groups (four rats per group). Two groups were treated with vehicle alone (control group); one group was used as the pre-treatment group in which rats were killed just before vehicle or MK treatment, and the others were used for MK-*n* treatment (MK-*n* groups). Normal rats fed a conventional diet were used for the normal group ($n = 8$). MK-*n* was suspended in 5% (w/v) gum arabic and adjusted to 20 mg/L. Rats in the MK-*n* groups were each given MK-*n* orally at a dose of 0.1 mg/kg body weight. Rats in the control group were given the same volume (5 mL/kg) of 5% (w/v) gum arabic solution orally. At 3 and 6 hr after administration, rats were anaesthetized with sodium pentobarbital (Nembutal®, Abbott Laboratories, North Chicago, IL, U.S.A.); 4.5 mL of blood was taken from the abdominal aorta by a syringe containing 0.5 mL of 3.8% (w/v) sodium citrate, and the liver was removed. Blood was centrifuged at 2600 *g* and 4° for 10 min and a plasma sample was prepared. Blood coagulation activity, normal prothrombin level, and MK-*n* levels in plasma were determined. The liver was washed in saline to remove blood and stored at −20° until the MK-*n* concentration was measured.

Experiment 2. Hypoprothrombinaemic and normal rats were prepared in the same manner as in experiment 1. MK-0, MK-4 and MK-9 were suspended in 5% (w/v) gum arabic and given orally to hypoprothrombinaemic rats at a dose of 0.1 mg/kg body weight. At 24 hr after administration, rats were killed under pentobarbital anaesthesia, and blood and livers were collected. Blood coagulation activity, normal prothrombin level in plasma and MK-*n* concentration in the liver were determined.

Experiment 3. Hypoprothrombinaemic and normal rats were prepared in the same manner as in experiment 1. Each MK-*n* was dissolved in 1% (w/v) polyoxyethylene 60 hydrogenated castor oil (Nikkol HCO-60®, Nikko Chem. Co., Japan). MK-*n* was injected into the tail veins of rats in the MK-*n* group at a dose of 10 nmol/kg body weight. Rats in control group were given 1 mL of 1% (w/v) HCO-60 solution/kg body weight, the same volume as the MK-*n* treatment. At 1 and 3 hr after treatment, rats were anaesthetized and plasma and livers were obtained in the same way as in experiment 1.

Chemical analyses. Blood coagulation time was measured by the Hepaplastintest® (Eisai Co. Ltd). Barium sulphate was added to pooled blood from normal rats to a concentration of 400 mg/mL, shaken for 20 min at room temperature, and the mixture centrifuged at 11,000 *g* for 3 min. $BaSO_4$-adsorbed and normal plasma were used as 0 and 100% extrinsic coagulation activity plasma, respectively. By mixing various proportions of the 0 and 100% activity plasma, 5, 10, 20, 30, 40, 60, and 80% activity plasma were prepared. The coagulation time of these plasma preparations was measured and a standard curve prepared by plotting coagulation time against blood coagulation activity, and the blood coagulation activity of the test sample was determined from this curve. Normal prothrombin in plasma was determined by the method of Shah *et al.* [13] as follows. To prepare $BaSO_4$-adsorbed plasma, 400 mg of $BaSO_4$ was added to 1 mL of plasma and shaken for 20 min at room temperature. Untreated and $BaSO_4$-adsorbed plasma samples were diluted 561- and 51-fold with 0.1 M Tris buffer pH 8.7, respectively. Venom (10 ng/mL, venom, Echis

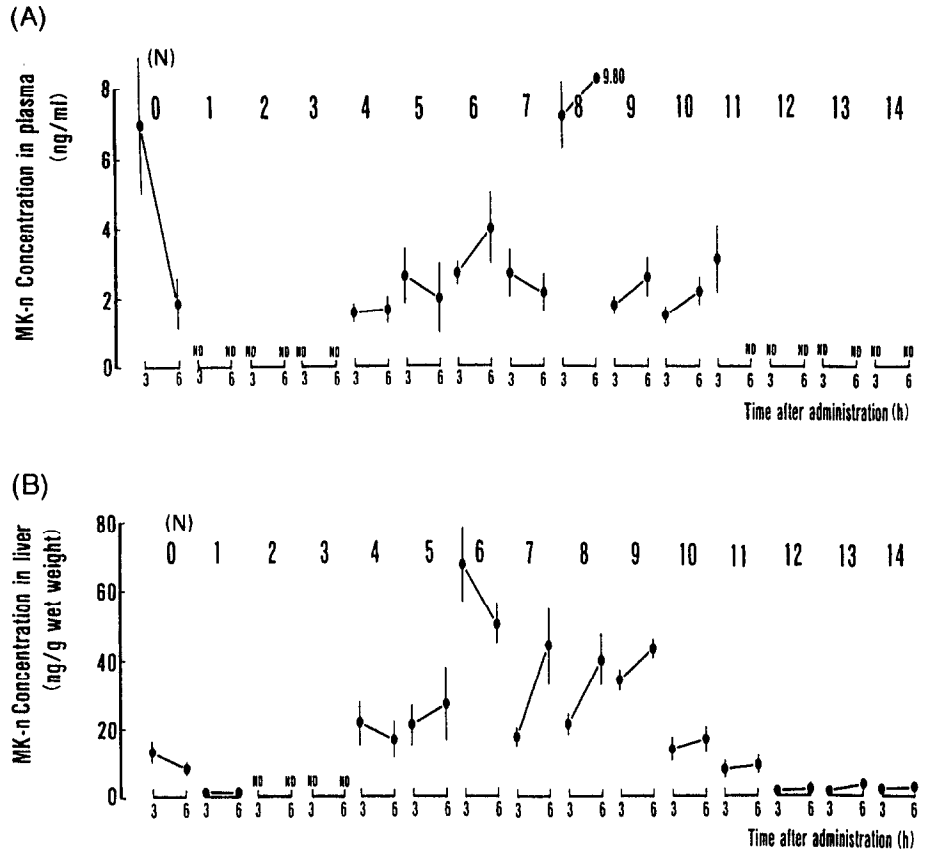

Fig. 2. Concentration of each MK-*n* in the plasma (A) and in the liver (B) 3 and 6 hr after oral administration. Each MK-*n* at a dose of 0.1 mg/kg body weight was administered orally to hypoprothrombinaemic rats induced by administration of a vitamin K-deficient diet and warfarin solution for 8 days. The mean dose of warfarin was 0.06 mg/kg body weight. MK-*n* was suspended in 5% (w/v) gum arabic. Each point and bar represent the mean ± SEM of four rats. N represents the number of isoprene units (0–14); ND, not detectable.

carinatus, Sigma, St. Louis, MO, U.S.A.) was added to each diluted plasma sample and incubated at 37° for 10 min. Then 3 mM of substrate (tosylglycyl-L-prolyl-L-arginine-*p*-nitroanilide acetate, Sigma, St. Louis, MO, U.S.A.) was added and the mixture incubated at 37°. After 10 min, the reaction was stopped by the addition of 20% (w/v) lauryl sulphate and the absorbance at 405 nm was measured. The results for the untreated and $BaSO_4$-adsorbed plasma represented total and abnormal prothrombin levels, respectively. Normal prothrombin was calculated from the difference between total and abnormal prothrombin levels.

The concentration of MK-*n* in the plasma and liver was measured as follows. Each MK-*n* was extracted with isopropyl alcohol and hexane and separated by HPLC [14]. The limits of detection of MK-*n* in plasma and liver were 0.45 pmol/mL and 0.45 pmol/g wet weight, respectively. Column: Nucleosil $5C_{18}$ 4 × 250 mm; reduction column: RC-10; elution buffer: methanol/ethanol (4:1, v/v) for MK-0 to MK-7, (1:4, v/v) for MK-8 to MK-14; fluorometric detection: Shimadzu RF-535, Ex 320 nm, E_m 430 nm.

Statistical analysis. All values are expressed as the means ± SEM. Significance of the difference between the control group and each MK-*n* group was determined by the Student's *t*-test.

RESULTS

Oral administration (experiments 1 and 2)

In these experiments, the activities of MK-*n* were compared on a weight basis, 0.1 mg/kg, which was 580 nmol/kg body weight (MK-0) to 89 nmol/kg body weight (MK-14) on a molar basis. Before MK treatment, the body weights of normal and hypoprothrombinaemic rats were 270 ± 8 g (N = 8) and 262 ± 1 g (N = 132), respectively. The concentrations of each MK-*n* in plasma 3 and 6 hr after oral administration of MK-*n* are shown in Fig. 2(A). No MK-*n* was detected in the plasma before the vehicle or MK-*n* treatment (pre-treatment group). In MK-0 and MK-4 to MK-11 groups (especially the MK-8 group) the concentrations of the homologues were increased in plasma at both 3 and 6 hr. The blood concentrations of MK-4 to MK-10 at 6 hr were almost the same as those at 3 hr.

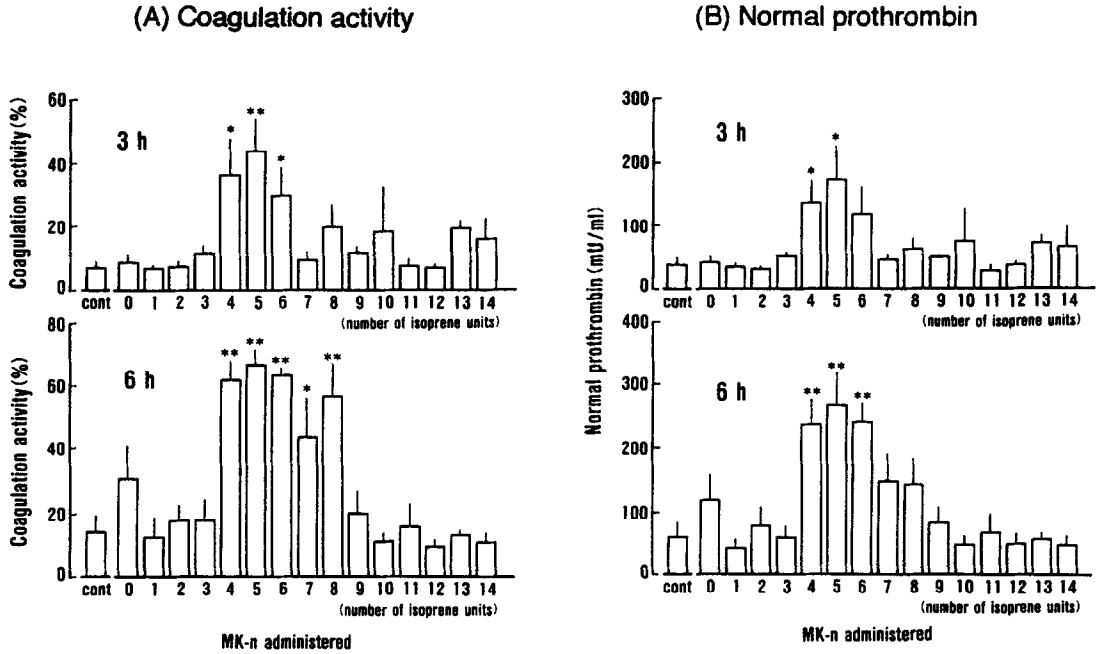

Fig. 3. The effects of oral MK-*n* administration (0.1 mg/kg) on blood coagulation activity (A) and the normal prothrombin level in plasma (B) 3 and 6 hr after administration. The conditions were the same as described in Fig. 2. Each column and bar represent the mean ± SEM of four rats. Significance of the difference between control and each MK-*n* was determined by the Student's *t*-test. $^{*}P < 0.05$, $^{**}P < 0.01$ versus control; cont: hypoprothrombinaemic rats given vehicle only.

In MK-0, the blood concentration at 3 hr was very high but was decreased at 6 hr. No MK-*n* was detected in the plasma in MK-1 to MK-3 and MK-12 to MK-14 groups at 3 or 6 hr.

The concentration of each MK-*n* in the liver 3 and 6 hr after oral administration is shown in Fig. 2(B). No MK-*n* was detected in the liver in the pre-treatment group. In MK-0 and MK-4 to MK-11 groups, the concentration of MK-*n* in the liver was increased, especially in MK-6, -7, -8 and -9, which were more than 40 ng/g wet weight at 6 hr. No MK-*n* was detected or less than 3 ng/g wet weight in the liver in MK-1 to MK-3 and MK-12 to MK-14 groups.

As shown in Fig. 3(A), blood coagulation activity in the hypoprothrombinaemic control group was 7.5–14.3% of that in the normal group. At 3 hr, blood coagulation activity in MK-4, -5 and -6 groups was significantly higher than that in the control group, and the activities in these groups were further increased at 6 hr. MK-7 and MK-8 increased coagulation activity significantly at 6 hr. Other MK-*n* did not affect this activity.

The effect of MK-*n* administration on normal prothrombin concentration in plasma is shown in Fig. 3(B). In the normal group, normal prothrombin concentration was 505 ± 16 mU/mL, whereas in the hypoprothrombinaemic control group it was 42 ± 10 mU/mL at 3 hr and 63 ± 23 mU/mL at 6 hr, indicating that rats in the control group had severe hypoprothrombinaemia. In the MK-4, MK-5 and MK-6 groups, normal prothrombin concentration was significantly higher than that in the control group 6 hr after administration.

Table 2 shows the effects of MK-0, MK-4 and MK-9 administration on blood coagulation activity, normal prothrombin concentration in plasma, and MK-*n* concentration in liver 24 hr after oral administration. All of these MK-*n*, especially MK-4, improved both blood coagulation activity and the normal prothrombin level in plasma compared with the control group.

Intravenous injection (experiment 3)

In this experiment, the activities of the MK-*n* were compared on a molar basis, 10 nmol/kg body weight. Before MK treatment, the body weights of normal and hypoprothrombinaemic rats were 239 ± 4 g (N = 8) and 233 ± 1 g (N = 132), respectively. The concentration of each MK-*n* in the liver 1 and 3 hr after intravenous injection of MK-*n* at 10 nmol/kg body weight is shown in Fig. 4. No MK-*n* was detected in the liver in the pre-treatment group. MK-0 concentration in the liver was very high, more than 100 pmol/g wet weight both 1 and 3 hr after treatment. Liver concentrations of MK-4 to MK-9 were more than 7.5 pmol/g wet weight. MK-1 and MK-2 were not detected in the liver 1 or 3 hr after treatment, and MK-12 was not detected at 3 hr.

Figure 5(A) shows the effect of MK-*n* on blood coagulation activity. At 1 hr, blood coagulation activity in the MK-4 and MK-5 groups was significantly higher than that in the control group.

Table 2. Effects of oral administration of MK-0, 4 and 9 on MK-*n* concentration in liver, blood coagulation activity and normal prothrombin level in plasma at 24 hr after administration

Group	N	Liver	Plasma	
		MK-*n* (ng/g wet weight)	Blood coagulation activity (%)	Normal prothrombin (mU/mL)
Normal	4		97.4 ± 2.6	499 ± 13
Hypoprothrombinaemic control	4		< 5.0	7 ± 3
MK-0	5	1.80 ± 0.18	43.0 ± 7.3	195 ± 23**
MK-4	5	2.08 ± 1.00	73.4 ± 17.0	404 ± 93**
MK-9	5	9.28 ± 1.51	29.1 ± 5.1	140 ± 21**

Each MK-*n* was suspended in 5% (w/v) gum arabic and 0.1 mg/kg was given orally to hypoprothrombinaemic rats induced by vitamin K-deficient diet and warfarin solution (0.39 mg/L) for 8 days. The mean daily dose of warfarin was 0.06 mg/kg body weight. Normal group: normal rats fed on conventional diet; control: hypoprothrombinaemic rats given vehicle only. Each value represents the mean ± SEM. ** $P < 0.01$ versus control (Student's *t*-test).

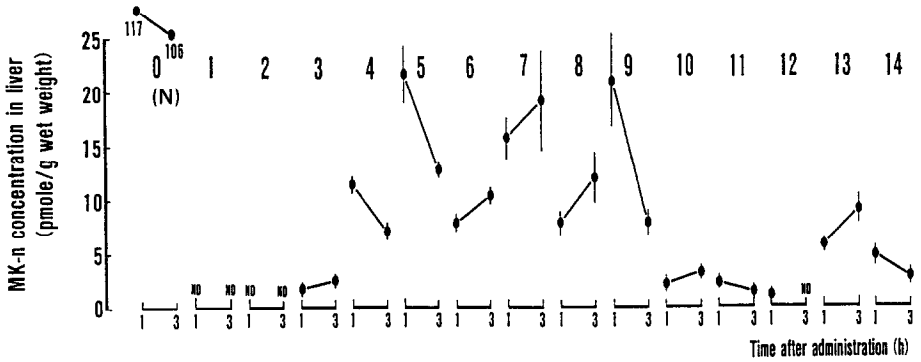

Fig. 4. Concentrations of each MK-*n* in the liver 1 and 3 hr after intravenous administration. MK-*n* was dissolved in 1% (w/v) of HCO-60 and MK-*n* at a dose of 10 nmol/kg body weight was given to hypoprothrombinaemic rats induced by administration of a vitamin K-deficient diet and warfarin solution for 8 days. The mean dose of warfarin was 0.06 mg/kg body weight. Each point and bar represent the mean ± SEM of four rats. ND, not detectable.

Activity in these two groups was further increased at 3 hr. In addition to MK-4 and -5, the MK-6 and -7 groups significantly increased blood coagulation activity at 6 hr compared with the control group. The effect of MK-*n* on normal prothrombin concentration in plasma is shown in Fig. 5(B). At 1 hr, normal prothrombin concentration in the MK-4 and MK-5 groups was significantly higher than that in the control group. In the MK-4, -5, -6, and -7 groups, the concentration was significantly higher than that in the control group at 3 hr.

DISCUSSION

The purpose of this study was to compare the physiological activities of MK-*n*. Therefore, severe hypoprothrombinaemia with high sensitivity to vitamin K and low inter-individual variation was required. Dietary deprivation of vitamin K in rats is reported not to induce severe hypoprothrombinaemia because of the supplementation of MK produced by intestinal flora [6, 15, 16] or coprophagy [17]. Therefore, hypoprothrombinaemia was induced by giving a diet deficient in vitamin K and a low dose of warfarin solution (0.06 mg/kg). Since warfarin inhibits vitamin K-epoxide reductase and vitamin K-reductase in the vitamin K cycle, resulting in the inhibition of γ-carboxylation of prothrombin precursors and a decrease in the normal prothrombin level in plasma [18], its administration induces a hypoprothrombinaemia similar to that induced by vitamin-K-deficient diet feeding. The inhibitory effect of MK homologue on these enzymes may influence the results on their physiological activities. However, since the dose of warfarin used was low and our findings are in good agreement with those reported by Matschiner *et al.* using vitamin-K-deficient animals [11], the ameliorating effects of MK on hypoprothrombinaemia are due to entering

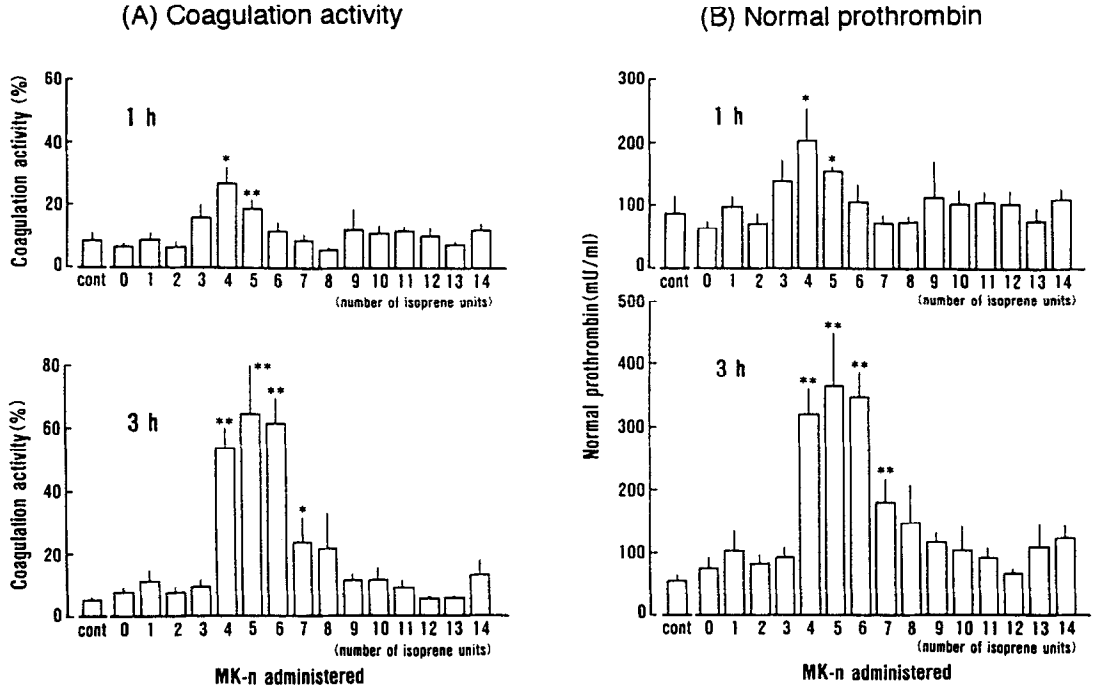

Fig. 5. The effects of intravenous MK-*n* administration (10 nmol/kg) on blood coagulation activity (A) and the normal prothrombin level in plasma (B), 1 and 3 hr after administration. The conditions were the same as described in Fig. 4. Each column and bar represent the mean ± SEM of four rats. Significance of the difference between control and each MK-*n* was determined by the Student's *t*-test. $^{*}P < 0.05$, $^{**}P < 0.01$ versus control; cont: hypoprothrombinaemic rats given vehicle only.

into the vitamin K-cycle and promoting γ-carboxylation of the prothrombin precursor.

MK, particularly those with multiple isoprene units, are synthesized by the intestinal flora: MK-7 to MK-14 (especially MK-10) by *Bacterioides fragilils*, and MK-6 to MK-9 (especially MK-8) by *Escherichia coli* [5, 6]. However, it is not clear whether MK synthesized by bacteria plays an important role in providing this vitamin to the host. Uchida *et al.* reported that bacterial populations are high in the large intestine [10], but since MK-*n* are absorbed from the upper part of the small intestine [19], these vitamins cannot be utilized efficiently to prevent vitamin K deficiency. In this study, 0.1 mg/kg body weight of MK with 0–14 isoprene units was given orally to hypoprothrombinaemic rats. MK-1 to MK-3 were not absorbed efficiently from the intestine after oral administration. In rats treated with MK-4 to MK-11, the molar base doses were from 225 nmol/kg to 109 nmol/kg, respectively, and the plasma and liver concentrations of each MK were significantly higher than those in the pre-treatment group (Figs 2 and 3). MK-4 to MK-9 were also detected in high concentrations in the liver after intravenous injection of 10 nmol/kg of the corresponding MK (Fig. 4, experiment 3). Thus, MK homologues absorbed from the intestine were similar to those detected in the liver. Although the molar base doses of MK with more than 12 isoprene units were not so different from those of MK with less than 11 isoprene units, MK-12 to MK-14 were not detected in the plasma after oral administration. These findings suggest that MK with more than 12 isoprene units are not readily absorbed from the intestine, even though they are produced by the intestinal flora. The fat solubility and micelle formation with bile acids of MK depend on the number of isoprene units, which may be related to the difficulty in absorption of MK with a longer side chain from the intestine.

MK-4 to MK-11 were found in the liver 3 hr after oral administration (Fig. 2(B)), but the elevation of MK-9 to MK-11 concentrations in the liver did not contribute to the improvement of hypoprothrombinaemia (Fig. 3). The same finding was obtained at 3 hr after 10 nmol/kg of MK injection; e.g. MK with 0, 8, 9, 13 and 14 isoprene units. These findings suggest that the physiological activity of MK in ameliorating vitamin K deficiency does not parallel its concentration in the liver. MK with 4–13 isoprene units was recovered in the human liver; the concentrations of MK with longer isoprene units of 7, 8, 10 and 11 were especially high [9] but contribute little to clotting factor synthesis [10]. These reports were consistent with our finding mentioned above. The reason why some MK which exist in the liver do not exert their activity is either that such MK may not be distributed in hepatocytes but to other cells in the liver (e.g. Kupper cells) or that these MK are hardly utilized in the vitamin K-cycle.

Some comparative studies of MK have been

reported. In *in vitro* studies measuring carboxylation activity, only MK with 1–3 isoprene units [20] or MK with 1–4 isoprene units [21] were compared. In *in vivo* studies, Matschiner *et al.* compared MK by bioassays in chicks [11] and in rats [12]. In the former study, MK-1 to MK-10 were given orally to chicks, and MK-4 to MK-10, especially MK-4, were found to be potent in ameliorating hypoprothrombinaemia. In their latter study using intracardial administration in rats, MK-7 to MK-10 were much more potent than MK-4. The difference between these two results might be due to the difference in administration route. Our findings are in good agreement with those obtained in chicks. We could not compare the exact relative potency of each MK-*n* because a dose–response study for each MK-*n* was not performed. However, we did examine the relationship between the number of isoprene units and vitamin K-activity, and obtained almost identical results in both oral and intravenous administration: MK with 4, 5, and 6 isoprene units quickly and potently ameliorated vitamin K-deficiency. Therefore, we believe that the difference in the results obtained in rats and in chicks by Matschiner *et al.* is not entirely due to the difference in administration route.

MK-9, a typical bacteria-produced MK, did not exhibit vitamin K activity 6 hr after oral administration. Therefore, we compared the vitamin K activity of MK-0, MK-4 and MK-9 at 24 hr after oral administration. MK-0 and MK-9 improved hypoprothrombinaemia 24 hr after treatment, but the potencies of MK-0 and MK-9 were less than that of MK-4 (Table 2). These findings suggest that the length of the isoprene unit affects the time taken to exert vitamin K activity. The finding that MK-0, 24 hr after oral administration, ameliorated hypoprothrombinaemia though its liver concentration was remarkably decreased compared with that 3 or 6 hr after treatment suggests that MK-0 exerts its physiological activity after the conversion to another active MK-homologue.

In conclusion, MK-4, MK-5 and MK-6 may be the most effective vitamin K_2 in nature, and MK with relatively longer isoprene units, and MK-7 to MK-13, synthesized by intestinal bacteria, are not efficiently absorbed, and even if absorbed are less effective in improving vitamin K-deficiency. As the results of oral administration on a weight basis were almost the same as those of intravenous administration on a molar basis, the different molar doses of MK in oral administration do not seriously affect the conclusion.

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