

New Developments in Research on Vitamin K Biosynthesis

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Phylloquinone (PK) is a major form of dietary vitamin K; however, the most prevalent form of vitamin K in humans and animals is menaquinone-4 (MK-4). Despite its high concentrations, the origin of MK-4 is yet to be defined. It is postulated that PK is converted into MK-4 and accumulates in extrahepatic tissues. To clarify this, PK with a deuterium-labelled 2-methyl-1,4-naphthoquinone ring was administered orally to mice and their cerebra were collected for deuterium (D) NMR and liquid chromatography (LC)-MS/MS analyses. We identified labelled MK-4 formed by conversion of the given PK, and this conversion occurred following oral or enteral administration but not parenteral or intracerebroventricular administration. Through the oral route, PK with the deuterium-labelled side chain in addition to the labelled 2-methyl-1,4-naphthoquinone was clearly converted into labelled MK-4 with a non-deuterium-labelled side chain, implying that PK was converted into MK-4 via the removal of an integral side chain. Our results suggest that cerebral MK-4 originates from PK intake, comprising the release of menadione (K_3) from PK in the intestine followed by prenylation of K_3 into MK-4 in the intestine or other tissues. We recently demonstrated that deuterium-labelled PK (PK- d_7) and deuterium-labelled K_3 (K_3 - d_8) are converted to deuterium-labelled MK-4 (MK-4- d_7) in human osteoblast-like MG-63 cells. Moreover, we identified that the side chain substrate involved in the conversion of PK or K_3 to MK-4 is geranylgeranyl diphosphate deriving from the mevalonate pathway. Therefore, MK-4 biosynthesis likely plays an important role in biological functions of the brain and bones.

Key words — vitamin K, menaquinone-4, mevalonate pathway, geranylgeranyl pyrophosphate

INTRODUCTION

Vitamin K is a cofactor for γ -glutamyl carboxylase (GGCX), an enzyme that converts specific glutamic acid residues in several substrate proteins to γ -carboxyglutamic acid (Gla) residues.^{1–3)} Gla residues form calcium-binding groups in proteins and are essential for their biologic activity. Gla-containing proteins are involved in blood coagulation,⁴⁾ bone metabolism,⁵⁾ vascular repair,⁶⁾ prevention of vascular calcification,⁷⁾ and regulation of cell proliferation and signal transduction.⁸⁾ Vitamin K undergoes a cyclic interconversion, the vitamin K cycle, comprising reduction of the vitamin K quinone form into hydroquinone, oxidation to 2,3-epoxide (vitamin K epoxide), and reduction

to quinone. The formation of Gla from glutamate is coupled with the conversion of hydroquinone to vitamin K epoxide. Both these activities occur for a cofactor of GGCX. The warfarin-sensitive microsomal enzyme, vitamin K epoxide reductase, recycles vitamin K epoxide back to hydroquinone, thus completing the vitamin K cycle.^{9–11)} Natural vitamin K exists in two molecular forms, phylloquinone [2-methyl-3-phytyl-1,4-naphthoquinone (PK); plant form] and menaquinones (MKs, bacterial forms).¹²⁾ All forms of vitamin K have a common 2-methyl-1,4-naphthoquinone (menadione; K_3) ring structure. At the 3-position of the ring, PK and MKs contain respectively a monounsaturated side chain of four isoprenoid residues and an unsaturated side chain with different number of isoprenoid residues.

PK is a major type of dietary vitamin K, although its concentrations in animals are markedly lower compared with MKs, in particular menaquinone-4 (MK-4). In 1958, Martius and Esser¹³⁾ first showed that 2-¹⁴C-methyl-

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1,4-naphthoquinone was converted to 2- ^{14}C -methyl-3-geranylgeranyl-1,4-naphthoquinone in chicks and rats. Martius' group^{14,15)} demonstrated in chickens and pigeons that PK doubly labelled with ^3H in the ring and ^{14}C in the side chain was converted to MK-4 singly labelled with ^3H . Using doubly labelled PK (with ^3H in the 2-methyl group of the naphthoquinone nucleus and ^{14}C in the side chain), they showed that the ^{14}C -labelled side chain of PK was cleaved and replaced with an unlabelled geranylgeranyl side chain. From these experiments, it was confirmed that both PK and MK-4 itself are endogenously converted to generate MK-4. With respect to the conversion site, the following two possibilities are proposed: conversion occurs in the gut by bacterial enzymatic activity or may occur in the intestine and/or local tissues. However, the former mechanism is unlikely as Roden *et al.*¹⁶⁾ and Davidson *et al.*¹⁷⁾ independently showed that conversion takes place in germ-free rats as well as in control animals. Therefore the latter mechanism is more likely and tissue MK-4 may originate from conversion of PK *in vivo*. This suggests that two pathways are possible for the conversion of PK to MK-4; first, side-chain removal occurs during intestinal absorption and released menadione is then transferred to tissues via the bloodstream and subsequently prenylated to form MK-4, and second, after transfer of PK to tissues, side-chain cleavage and geranylgeranylation occur simultaneously therein. Thijssen *et al.*¹⁸⁾ reported that urinary menadione excretion increases after intake of single doses of oral PK and mirrored PK in a 30-day depletion/repletion human metabolic study, together implying that menadione may be an intermediate in the conversion of PK to MK-4.

Our recent report¹⁹⁾ indicates that cerebral MK-4 originates not only from systemic conversion, comprising the release of K_3 from PK in the intestine and the prenylation of K_3 into MK-4 in cerebrum, but also from the in-cell conversion of PK into MK-4 in cerebrum.¹⁹⁾ We used stable isotope-labelled vitamin K compounds, which are particularly useful for distinguishing the behavior of exogenous compounds from that of the corresponding endogenous compounds on the basis of structural assignments determined by NMR spectrometry and liquid chromatography (LC)-MS/MS. We synthesized deuterium (D)- or heavy oxygen (^{18}O)-labelled forms of PK and MK-4 in our laboratory. Using these compounds, we were able to obtain unequivocal evidence regarding the origin of MK-4

in cerebra of mice. Our findings suggest that MK-4, a transcriptional regulator of steroid and xenobiotic receptor (SXR)-mediated signalling²⁰⁾ as well as cofactor for GGCX, is not simply a dietary nutrient but should also be regarded as an active form of vitamin K that may contribute to neural functions in mammals. In our previous study, conversion of deuterium-labelled PK (PK-d₇) and deuterium-labelled K_3 (K_3 -d₈) to deuterium-labelled MK-4 (MK-4-d₇) occurred in human osteoblast-like MG-63 cells.²¹⁾ Moreover, we identified that the side-chain substrate for conversion of PK or K_3 to MK-4 is geranylgeranyl diphosphate (GGPP) derived from the mevalonate pathway.²¹⁾ In this minireview, we report our recent study of MK-4 biosynthesis.

MK-4 BIOSYNTHESIS IN MOUSE

Stable isotope-labelled compounds are particularly useful for distinguishing the behaviour of exogenous compounds from that of the corresponding endogenous compounds on the basis of structural assignments by NMR spectrometry and LC-MS/MS. We synthesized D- or ^{18}O -labelled forms of PK and MK-4 in our laboratory (Fig. 1). Using these compounds, we were able to obtain unequivocal evidence regarding the origin of MK-4 in cerebrum of mice. We examined whether orally administered PK-d₇ accumulates in cerebrum as a converted form of MK-4-d₇. Two hundred female mice were orally administered PK-d₇

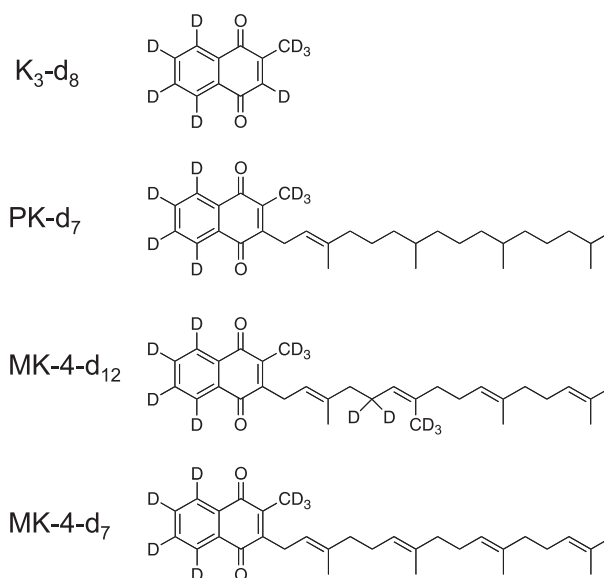


Fig. 1. Chemical Structures of Deuterium (D)-labelled Vitamin K Derivatives

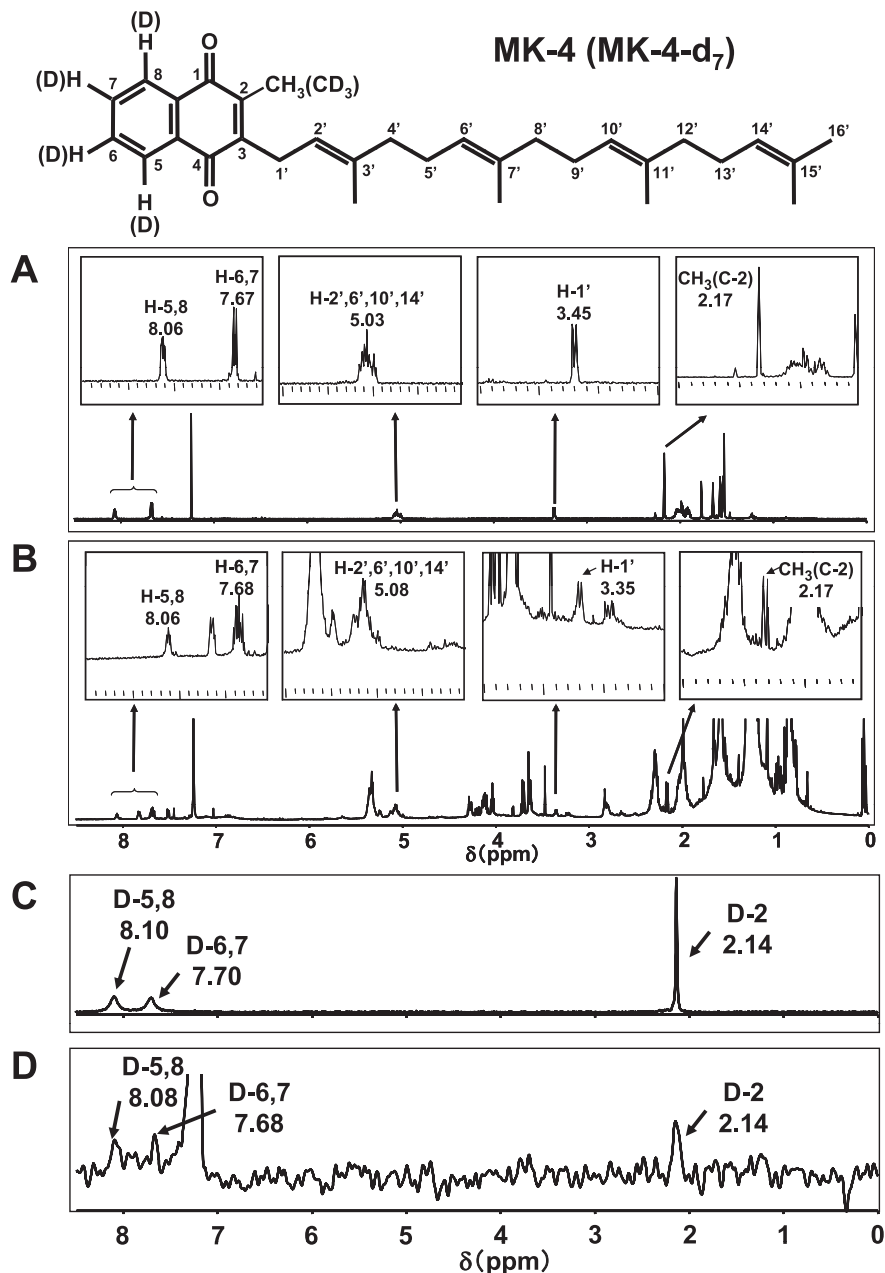


Fig. 2. ^1H NMR and D NMR Analyses of MK-4 Fraction Purified from Cerebra of Mice Orally Administered PK- d_7^{19}

A and B, ^1H NMR spectra of authentic MK-4 and MK-4 fraction, respectively. C and D, D NMR spectra of authentic MK-4- d_7 and MK-4 fraction, respectively. The number and letters (H, D) in each spectrum refer to the chemical shift (ppm) and the respective position of the proton and D in 2-methyl-1,4-naphthoquinone ring or side chain of MK-4 and MK-4- d_7 .

at a single dose of $10\text{ }\mu\text{mol/kg}$ body weight, and cerebra were collected at 24 hr after administration. After purification by HPLC, the MK-4 fraction was obtained; this contained MK-4 and MK-4- d_7 in amounts of 3.8 and 2.0 mg, respectively. Because tissue MK-4 has not yet been identified on the basis of structural assignments in humans and animals, and it is not known whether purified MK-4- d_7 in the presence of endogenous MK-4 can be identified by D NMR, we analyzed the MK-4 fraction by ^1H NMR spectroscopy. Conse-

quently, the values of resonance derived from the 2-methyl-1,4-naphthoquinone ring and the geranylgeranyl side chain of the MK-4 fraction entirely coincided with those of authentic MK-4. The values of resonance derived from the D-labelled 2-methyl-1,4-naphthoquinone ring of the MK-4 fraction exactly coincided with those of authentic MK-4- d_7 (Fig. 2). In LC-atmospheric pressure chemical ionization (APCI)-MS/MS analyses, authentic MK-4 gave a parent peak (Q1) at m/z 445.6 $[\text{M}+\text{H}]^+$ and a product ion peak (Q3) at m/z 187.3 [2,3-dimethyl-

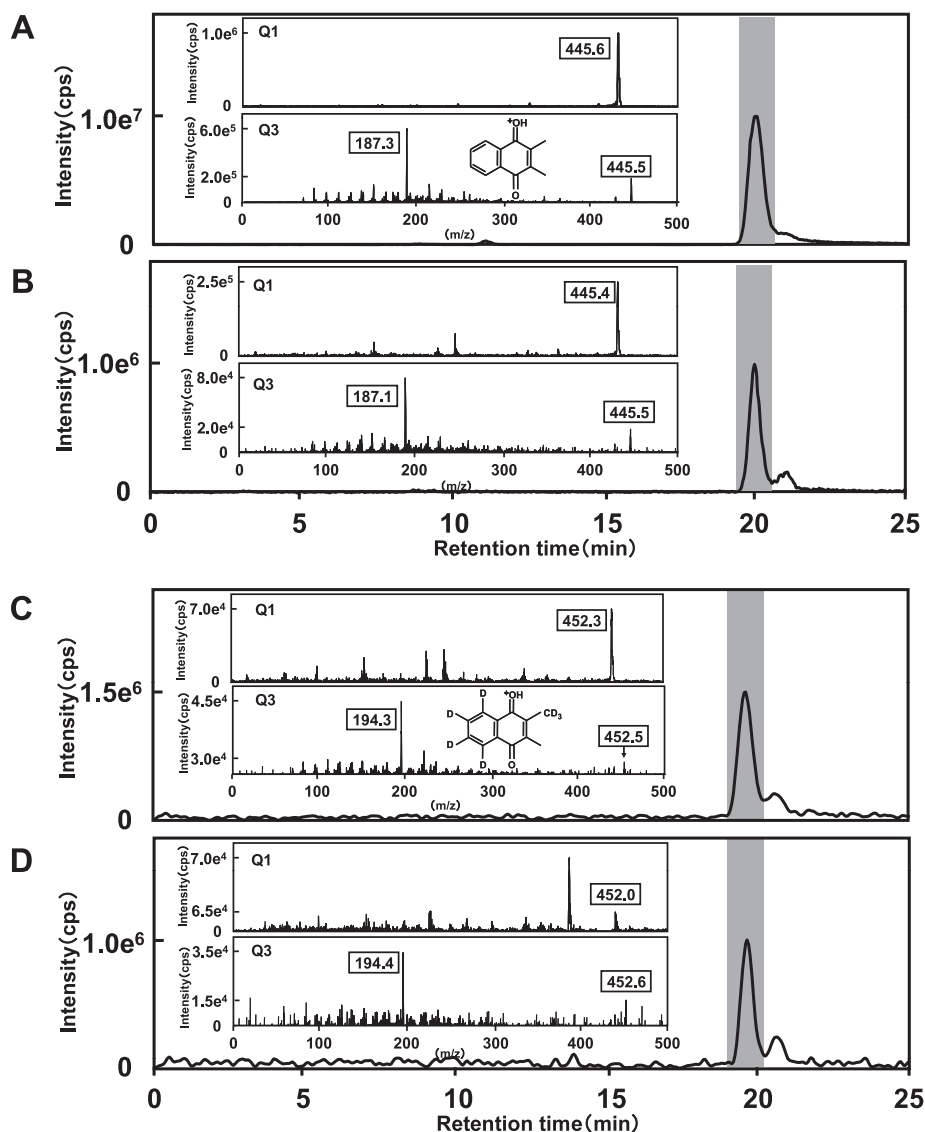


Fig. 3. LC-APCI-MS/MS Analyses of MK-4 Fraction Purified from Cerebra of Mice Orally Administered PK-d₇¹⁹⁾

A and B, MRM chromatograms and MS spectra of authentic MK-4 and MK-4 fraction (m/z 445.4 for MK-4), respectively. The parent ion MS spectra (Q1) and product ion MS spectra (Q3) derived from (Q1) were recorded at the time corresponding to the top of the peak (shaded area) on the MRM chromatograms. Similarly, C and D, MRM chromatograms and MS spectra of authentic MK-4-d₇ and MK-4 fraction (m/z 452.0 for MK-4-d₇), respectively. The parent ion MS spectra (Q1) and product ion MS spectra (Q3) derived from (Q1) were recorded as noted above.

1,4-naphthoquinone +H⁺)⁺. The MK-4 fraction gave the same parent peak at m/z 445.4 and a product ion peak at m/z 187.1. Authentic MK-4-d₇ gave a parent peak (Q1) at m/z 452.3 and a product ion peak (Q3) at m/z 194.3 [2,2,2,5,6,7,8-deuterated 2,3-dimethyl-1,4-naphthoquinone +H⁺)⁺. The MK-4 fraction gave the same parent peak at m/z 452.0 and a product ion peak at m/z 194.4. The LC-APCI-MS/MS multiple reaction monitoring (MRM) chromatograms and MS spectra of the MK-4 fraction were completely congruent with those of authentic MK-4 and MK-4-d₇ (Fig. 3). Thus based on the results of the ¹H NMR, D NMR, and LC-APCI-

MS/MS analyses it is evident that MK-4 exists in cerebra of mice and originates from intake of PK.

To obtain insight into the metabolic sites where PK is converted into MK-4, mice were orally, enterally, or intravenously administered PK-d₇ or K₃-d₈ as a single dose of 10 μmol/kg body weight, or were intracerebroventricularly administered PK-d₇ or K₃-d₈ at a dose of 0.1 μmol/kg body weight. After 24 hr, the concentrations of MK-4-d₇ and its epoxide in cerebra were measured by LC-APCI-MS/MS. With respect to the oral route, both PK-d₇ and K₃-d₈ induced accumulation of MK-4-d₇ or its epoxide, and conversion of PK-d₇ to MK-4-d₇ was

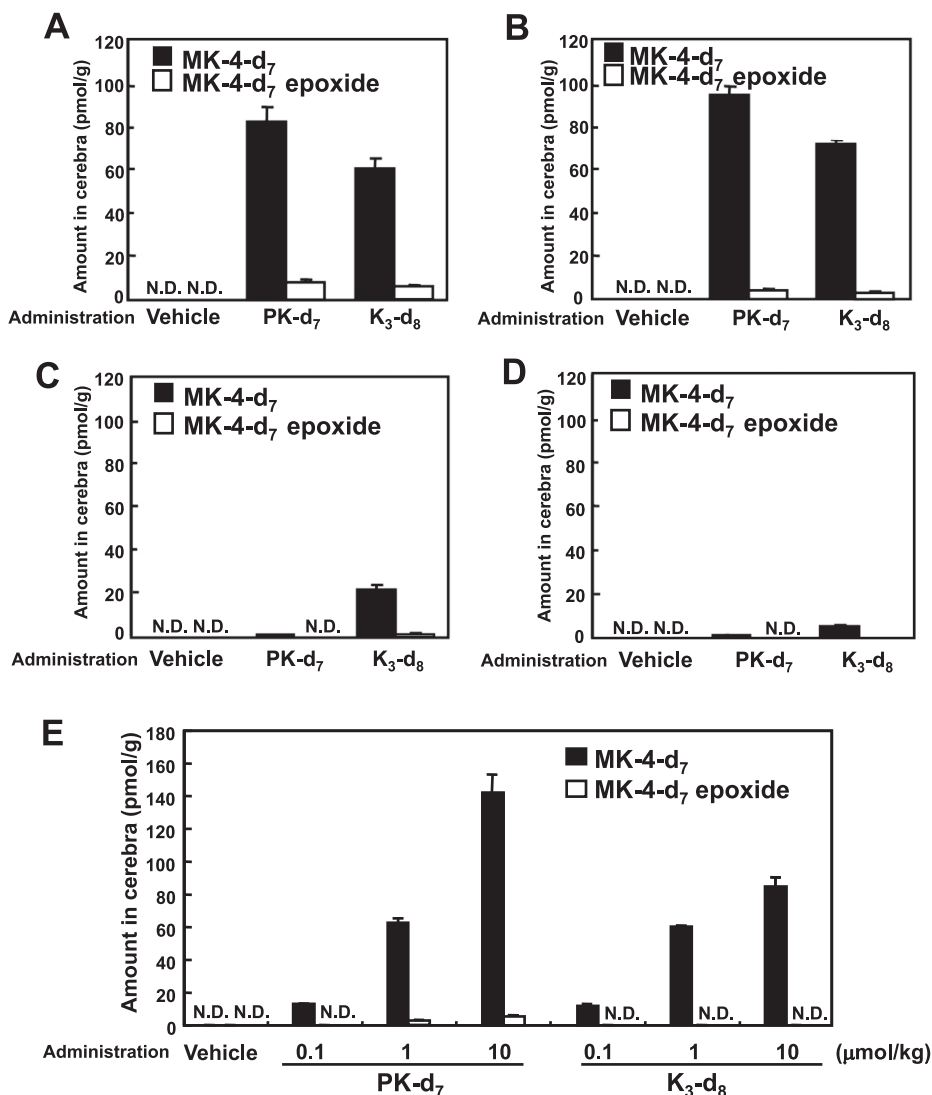


Fig. 4. Accumulation of MK-4-d₇ and MK-4 Epoxide-d₇ in Cerebra of Mice Administered PK-d₇ or K₃-d₈ via Four Routes¹⁹⁾

A, oral route; B, enteral route; C, intravenous route; and D, intracerebroventricular route. Female 8-week-old mice were fasted for 12 hr preadministration, then orally, enterally, or intravenously administered PK-d₇ or K₃-d₈ (10 μ mol/kg body weight) or intracerebroventricularly administered PK-d₇ or K₃-d₈ (0.1 μ mol/kg body weight). After 24 hr, the mice were sacrificed and their cerebra collected to measure MK-4-d₇ and its epoxide by LC-APCI-MS/MS as described under "Experimental Procedures." Results represent the mean for five mice (values in columns) and standard errors (vertical bars). N.D., undetectable on MRM chromatogram.

as efficient as that of K₃-d₈ to MK-4-d₇ (Fig. 4A). With respect to the enteral route, both PK-d₇ and K₃-d₈ induced accumulation of MK-4-d₇ and its epoxide; as expected, the efficiency with which PK-d₇ was converted to MK-4-d₇ was similar to that observed by the oral route (Fig. 4B). These results indicate that release of K₃ from PK does not necessarily require the aid of gastric juices. With respect to the intravenous route, K₃-d₈ induced accumulation of MK-4-d₇ and its epoxide at low levels, but PK-d₇ did not (Fig. 4C). With respect to the intracerebroventricular route, K₃-d₈ induced accumulation of MK-4-d₇ and its epoxide at low levels, but PK-d₇ did not (Fig. 4D). To examine whether the

conversion of PK-d₇ or K₃-d₈ to MK-4-d₇ occurs in a physiological state, we carried out a dose-response study by the oral route and confirmed that both PK-d₇ and K₃-d₈ were linearly converted to MK-4-d₇ and MK-4-d₇ epoxide (Fig. 4E).

CONVERSION OF PK-d₇ OR K₃-d₈ TO MK-4-d₇ IN MOUSE CEREBRAL SLICE AND PRIMARY CULTURES

To clarify whether PK and K₃ are converted directly to MK-4 without the aid of intestinal absorption and tissue distribution processes, mice were

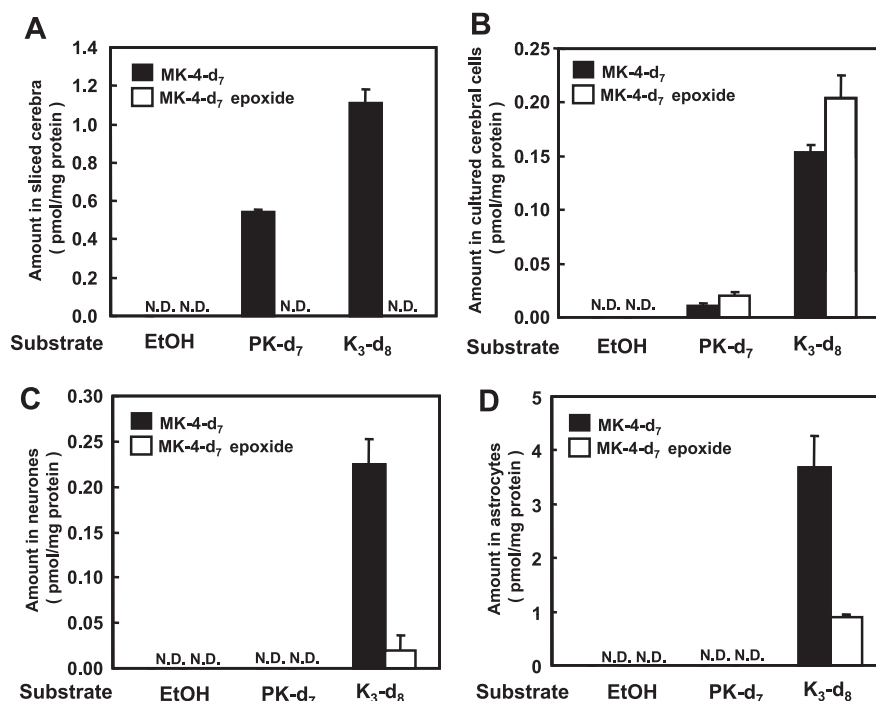


Fig. 5. Conversion of PK-d₇ or K₃-d₈ to MK-4-d₇ in Mouse Cerebellar Slice and Primary Cultures¹⁹⁾

A, cerebellar slice culture; B, cerebellar primary culture; C, neurons; and D, astrocytes. Sliced cerebra were incubated with either vehicle (ethanol), 10⁻⁵ M PK-d₇, or 10⁻⁵ M K₃-d₈ for 24 hr. Cerebellar primary cultured cells, neurons, and astrocytes were incubated with either vehicle (ethanol), 10⁻⁶ M PK-d₇, or 10⁻⁶ M K₃-d₈ for 24 hr. After incubation, lipids were extracted to measure MK-4-d₇ and MK-4 epoxide-d₇ by LC-APCI-MS/MS as described under "Experimental Procedures." Results represent means of three experiments (values in columns) and standard errors (vertical bars). N. D., undetectable on MRM chromatogram.

sacrificed and their cerebra excised to make slice cultures. Sliced cerebra were incubated with either PK-d₇ or K₃-d₈ at 10⁻⁵ M for 24 hr, and MK-4-d₇ and its epoxide were measured by LC-APCI-MS/MS. Surprisingly, both PK-d₇ and K₃-d₈ caused remarkable accumulation of MK-4-d₇ in cerebra (Fig. 5A), suggesting that release of K₃-d₇ from PK-d₇ and geranylgeranylation of K₃ occur within cerebra. Taken together, it is evident that the cerebrum itself has enzyme(s) or biological machinery for cleaving the phytyl side chain of PK and introducing the geranylgeranyl group to position 3 of the K₃ molecule. Considering this result, we examined whether conversion takes place in cerebral primary culture. As shown in Fig. 5B, a small but appreciable amount of MK-4-d₇ was detected and the K₃-d₈ to MK-4-d₇ conversion was much more prominent, although both conversion efficiencies in the primary culture were apparently lower than those observed in slice cultures. Another interesting feature was that both PK-d₇ and K₃-d₈ generated substantial amounts of MK-4-d₇ epoxide, which exceeded the levels of MK-4-d₇, probably due to the enhanced turnover rate of the vitamin K cycle in the primary culture. To examine the types of neuronal

cells responsible for this conversion, we prepared primary cultures with a high density of neurons or astrocytes, and performed the same conversion experiment as above. As shown in Fig. 5C and 5D, no conversion of PK-d₇ to MK-4-d₇ was detected, whereas conversion of K₃-d₈ to MK-4-d₇ occurred as efficiently in the primary culture as in the neuronal cell cultures. In neuron or astrocyte cultures, conversion of K₃-d₈ to MK-4-d₇ occurred with high efficiency. These results suggest that both neurons and astrocytes lack the ability to convert PK to MK-4, but they have the ability to prenylate K₃ into MK-4. The efficiency with which K₃ was converted to MK-4 was remarkably higher in astrocytes than in neurons.

CONVERSION OF K₃-d₈ TO MK-4-d₇ IN HUMAN OSTEOBLAST-LIKE MG-63 CELLS

In mice, PK-d₇ and D-labelled K₃-d₈ administration led to high levels of tissue MK-4-d₇, particularly in bone. We examined conversion of PK-d₇ and K₃-d₈ into MK-4-d₇ in human osteoblast-like

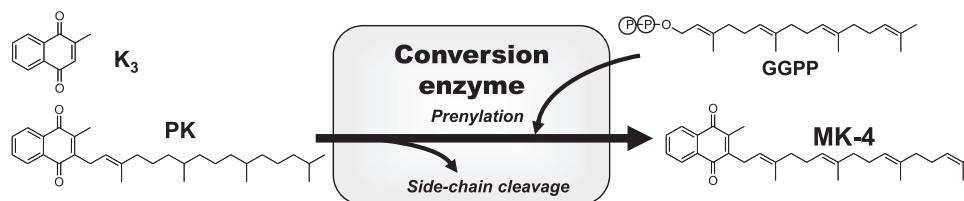


Fig. 6. Scheme Showing Conversion of Vitamin K Derivatives to MK-4

MG-63 cells. In *in vitro* assay, K₃-d₈ was converted into MK-4-d₇ more efficiently than PK-d₇. It is postulated that conversion of PK into MK-4 is a metabolic process involving enzymes responsible for cleavage of the side chain of PK and subsequent prenylation of K₃ (Fig. 6). There are conflicting reports with regard to the tissue sites of K₃ release. Using HPLC analysis, Thijssen *et al.*²²⁾ found that various cell lines were capable of converting K₃ into MK-4, but none was able to convert PK into MK-4, presumably due to a lack of K₃-releasing activity. On the other hand, Davidson *et al.*²³⁾ reported that human embryonic kidney (HEK) 293 cells converted PK into MK-4 epoxide. In the present study, PK-d₇ to MK-4-d₇ conversion did occur in mouse *in vivo* assay. However, osteoblasts were able slightly to convert PK-d₇ into MK-4-d₇. The reason for these conflicting results is still unclear, but one explanation may be that the PK to MK-4 conversion in mouse bone occurs at selective sites via selective pathways, including the intestine, liver, or several digestive organs. At present, we have no evidence related to these putative enzymes; further studies are required to define and characterize them. MK-4 plays a key role in bone homeostasis and is a clinically effective therapeutic agent for osteoporosis. MK-4 is a transcriptional regulator of bone marker genes in osteoblasts and potentiates bone formation by activating the steroid and xenobiotic receptor SXR.²⁰⁾ Other reports have previously not indicated the transcriptional activity and GGCX activity of MK-4 converted from PK or K₃. In this report, we first demonstrated that MK-4 converted from K₃ could induce SXR-dependent transcriptional activity in MG-63 cells. These results suggest that bone MK-4 originates not only from systemic conversion, comprising the release of K₃ from PK in the intestine and the prenylation of K₃ into MK-4 in the bone, but also from the in-cell conversion of K₃ into MK-4 in osteoblasts. Our findings indicate that MK-4, a transcriptional regulator of SXR-mediated signalling as well as cofactor for GGCX, is not simply a dietary nutrient but

should also be regarded as an active form of vitamin K that may contribute to bone formation in mammals.

MEVALONATE PATHWAY AND MENAQUINONE-4 BIOSYNTHESIS

GGPP is an intermediate product in the mevalonate pathway, which provides the only source of this compound in mammals. GGPP plays decisive roles in cell signalling and its presence is crucial to cell survival.²⁴⁾ GGPP synthesis appears tightly regulated within the mevalonate pathway, especially by the rate-limiting activity of hydroxymethylglutaryl-coenzyme A reductase (HMG-CoA reductase). Farnesylpyrophosphate (FPP) formation is the branch point of this pathway, serving as a precursor of cholesterol and its products and as an immediate precursor of GGPP.²⁵⁾ FPP and GGPP provide the basis for all longer-chain isoprenoids such as heme A, dolichol, and ubiquinone. Menaquinone is derived from the common branch point chorismate intermediate of the shikimate pathway in bacteria. Biosynthesis of menaquinone has been well-characterized in *Escherichia coli* (*E. coli*). The menaquinone biosynthesis pathway in *E. coli* established that the naphthoquinone nucleus was formed from shikimate (chorismate), and that isoprenoid side chains were derived from isoprenoid alcohol pyrophosphate ester in the mevalonate metabolic pathway. It was previously considered that humans do not synthesize menaquinone but must obtain it from diet or bacteria present in the gut. However, we demonstrated that MK-4 is synthesized from K₃ in mouse and human cells. The side chain of MK-4 was predicted to be derived from GGPP in the mevalonate pathway in mouse and human cells. Therefore we elucidated the conversion reaction using K₃-d₈ and D-labelled GGPP (GGPP-d₅) with LC-APCI-MS/MS. We used deuterated compounds so as to distinguish them from the native MK-4, K₃, and GGPP originally contained in the cell and to

observe the synthetic reaction itself. The aim of our study was to confirm the synthesis of D-labelled MK-4 (MK-4-d₁₂) in human osteoblast-like MG-63 cells using K₃-d₈ and GGPP-d₅. When ethanol, K₃-d₈, or GGPP-d₅ alone was added MK-4-d₁₂ was not detected at all. When 1 or 10 μM of K₃-d₈ and GGPP-d₅ were added at the same time, MK-4-d₁₂ was produced in a dose-dependent manner.²¹⁾ This result indicates that MK-4 could be converted from K₃ by prenylation with GGPP. In this study, we examined the mechanism by which MK-4 is converted in osteoblastic cells using deuterated compounds and vitamin K derivatives. These results indicate that the prenyl side chain of MK-4 is derived from GGPP and is connected to K₃ by a specific side-chain transfer enzyme.

CONCLUSION

Our recent studies show for the first time that MK-4-d₇ existing in the cerebrum of mice originates from intake of PK-d₇ and/or K₃-d₈, and that osteoblasts can convert K₃ to MK-4. We discovered for the first time that GGPP is a side-chain substrate for MK-4 biosynthesis in mammals. MK-4 converted from K₃ can activate SXR-regulated gene expression for bone formation in osteoblasts. The present results, which are derived both from structural data and biochemical characterization of animals as well as cell cultures or protein and genetic data, may aid in clarification of the physiological role of MK-4 in mammals and in the development of new drugs for bone homeostasis and the treatment of various diseases.

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