

New Development of Vitamin K Research: Identification of Human Menaquinone-4 Biosynthetic Enzyme

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ABSTRACT

Natural vitamin K is found in two forms: a plant form, phylloquinone (PK) and bacterial forms, menaquinones (MKs). In many species, including humans, PK is a minor constituent of hepatic vitamin K content, with most hepatic vitamin K content comprising long-chain MKs. Menaquinone-4 (MK-4) is ubiquitously present in extrahepatic tissues, with particularly high concentrations in the brain, kidney and pancreas of humans and rats. It has consistently been shown that PK is endogenously converted to MK-4. The molecular mechanisms for these conversion reactions have been unclear. To identify the MK-4 biosynthetic enzyme, we screened the human genome database for prenylation enzyme. We found UbiA prenyltransferase domain containing 1 (UBIAD1), a human homologue of *Escherichia coli* prenyltransferase menA. The short interfering RNA against the UBIAD1 gene inhibited the conversion of deuterium-labelled vitamin K derivatives into deuterium-labelled-MK-4 (MK-4-d₇) in human cells. We confirmed that the UBIAD1 gene encodes an MK-4 biosynthetic enzyme through its expression and conversion of deuterium-labelled vitamin K derivatives into MK-4-d₇ in insect cells infected with UBIAD1 baculovirus. UBIAD1 was localized in endoplasmic reticulum. Our results show that UBIAD1 is a human MK-4 biosynthetic enzyme; this identification will permit more effective decisions to be made about vitamin K intake and bone health.

Key words: vitamin K, phylloquinone, menaquinone-4, conversion, UBIAD1

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 serve to 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⁾, 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 the hydroquinone, oxidation to 2,3-epoxide (vitamin K epoxide), and reduction to the quinone. The formation of Gla from glutamate is coupled with the conversion of the hydroquinone to the vitamin K epoxide. Both of these activities occur in GGCX. The warfarin-sensitive microsomal enzyme, vitamin K epoxide reductase, recycles the vitamin K epoxide back to the hydroquinone, thus completing the vitamin K cycle⁽⁹⁻¹¹⁾. The metabolic requirement for vitamin K is satisfied by dietary phylloquinone (2-methyl-3-phytyl-1,4-naphthoquinone: PK) present in plants and, to an undetermined extent, by a bacterially produced series of 2-methyl-3-polyisoprenyl homologues called menaquinones (MK-n) produced in

the lower bowel⁽¹²⁾. Menadione (vitamin K₃: K₃), commonly used in animal diets, is converted to 2-methyl-3-geranylgeranyl-1,4-naphthoquinone (MK-4) when administered to animals⁽¹³⁾. A remarkable observation in vitamin K disposition is the conversion of PK into MK-4. Several reports showed that MK-4 accumulates in the tissues of rats following the administration of PK⁽¹⁴⁾. MK-4 was detected at low levels in the plasma and liver, and at much higher levels in the extrahepatic tissues such as the tissues in brain, pancreas, thyroid gland, kidney and bone. It has been well known since the 1960s that both K₃ and PK are converted to MK-4 in rats and chickens. Recent studies using germ-free rats showed that the conversion of PK to MK-4 was independent of the gut flora^(15,16). However, neither the pathway nor the function of the conversion PK into MK-4 is known. At the moment, two routes can be supposed: first, desaturation of the phytyl side chain to produce the geranylgeranyl group of MK-4; second, removal of the phytyl side chain to release K₃, which is subsequently prenylated. Our recent report indicates that cerebral MK-4 originates from not only systemic conversion comprising the release of K₃ from PK in the intestine and the prenylation of K₃ into MK-4 in the cerebra but also the in-cell conversion of PK into MK-4 in cerebra⁽¹⁷⁾. We used the stable isotope-labeled vitamin K compounds. Stable isotope-labeled compounds are

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particularly useful for distinguishing the behavior of exogenous compounds from that of the corresponding endogenous compounds on the basis of structural assignments by nuclear magnetic resonance (NMR) spectrometry and liquid chromatography-tandem mass spectrometry (LC-MS/MS). We synthesized deuterium (D)- or heavy oxygen (^{18}O)-labeled forms of PK and MK-4 in our laboratory. Using these compounds, we were able to obtain unequivocal evidence of the origin of MK-4 in the cerebra of mice. Our findings suggest that MK-4, a transcriptional regulator of steroid and xenobiotic receptor (SXR)-mediated signaling⁽¹⁸⁾ as well as a cofactor for GGX, is not simply a dietary nutrient, but should be regarded as an active form of vitamin K that may contribute to neural functions in mammals. In our previous study, the PK-d₇ to MK-4-d₇ conversion indeed occurred in the cerebral slice culture as well as primary culture, however, neither neurons nor astrocytes converted PK-d₇ into MK-4-d₇⁽¹⁷⁾. The reason for these conflicting results is as yet unclear.

Recently, we succeeded to identify a human MK-4 biosynthetic enzyme. We screened the human genome database for prenylation enzymes and found UbiA prenyltransferase containing 1 (UBIAD1), a human homologue of *Escherichia coli* prenyltransferase menA. We found that short interfering RNA against the UBIAD1 gene inhibited the conversion of deuterium-labelled vitamin K derivatives into deuterium-labelled-MK-4 (MK-4-d₇) in human cells. We confirmed that the UBIAD1 gene encodes an MK-4 biosynthetic enzyme through its expression and conversion of deuterium-labelled vitamin K derivatives into MK-4-d₇ in insect cells infected with UBIAD1 baculovirus⁽¹⁸⁾. UBIAD1 was localized in endoplasmic reticulum and ubiquitously expressed in several tissues of mice. Our results show that UBIAD1 is a human MK-4 biosynthetic enzyme; this identification will permit more effective decisions to be made about vitamin K intake and bone health.

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. 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 cerebra as a converted form of MK-4-d₇. Two hundred female mice were orally administered PK-d₇ at a single dose of 10 $\mu\text{mol/kg}$ body weight, and cerebra were collected at 24 h after administration. After purification by HPLC, the MK-4 fraction was obtained; this contained MK-4 and MK-4-d₇ 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-d₇ in the presence of endogenous MK-4 can be identified by D NMR, we analyzed the MK-4 fraction by ^1H NMR spectroscopy. Consequently, 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-d₇. The LC-APCI-MS/MS MRM chromatograms and MS spectra of the MK-4 fraction were completely congruent with those of authentic MK-4 and MK-4-d₇. 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 $\mu\text{mol/kg}$ body weight, or were intracerebroventricularly administered PK-d₇ or K₃-d₈ at a dose of 0.1 $\mu\text{mol/kg}$ body weight. After 24 h, 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 as efficient as that of K₃-d₈ to MK-4-d₇. 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. 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. 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. 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.

CONVERSION OF PK AND K₃ TO MK-4 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 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. 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⁽¹⁹⁾. Our findings indicate that MK-4, a transcriptional regulator of SXR-mediated signalling as well as cofactor for GGXX, 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.

IDENTIFICATION OF MK-4 BIOSYNTHETIC ENZYME

In *E. coli*, MKs are involved in several anaerobic electron transport systems⁽²⁰⁾; it is the major transporter of electrons under anaerobic growth conditions⁽²¹⁾. Six genes (*menA*, *menB*, *menC*, *menD*, *menE* and *menF*) are involved in the biosynthetic pathway of MKs, and a key reaction for menaquinone biosynthesis in *E. coli* is the conversion of 1,4-dihydroxy-2-naphthoic acid (a bicyclic naphthalenoid) to the membrane-bound demethylmenaquinone. The key enzyme catalysing this reaction is encoded by *menA*⁽²¹⁾. Several human homologues of the *E. coli* menaquinone biosynthetic enzymes exist in the human genome database. However, the biological functions of these homologous genes remain unknown. UBIAD1 was the first identified mammalian homologue of *E. coli* *menA*. In *E. coli*, the *menA* gene encodes a prenyltransferase that is involved in the vitamin K biosynthetic pathway⁽²¹⁾. However, the function of human UBIAD1 has not been determined. Other prenylation enzymes, UbiA in *E. coli* and COQ2 in the yeast *Saccharomyces cerevisiae*, catalyse the prenylation of p-hydroxybenzoate, a critical step in ubiquinone biosynthesis.

In humans this step is catalysed by human COQ2, a coenzyme Q2 homologue of prenyltransferase (in yeast)⁽²⁴⁾. The low sequence homology between the UBIAD1 and COQ2 genes imply a different role for UBIAD1. We therefore predicted that either UBIAD1 or COQ2 is a prenylation enzyme for MK-4 biosynthesis in humans. To check this hypothesis, we used short interfering RNA (siRNA) against UBIAD1 and COQ2 genes to test their abilities to inhibit the conversions of K₃-d₈ and MK-4-d₁₂ into MK-4-d₇ in MG-63 cells. In contrast with COQ2 siRNA, UBIAD1 siRNA strongly decreased the conversions of K₃-d₈ and MK-4-d₁₂ into MK-4-d₇. We also constructed a UBIAD1 expression vector and analysed the K₃-d₈ into MK-4-d₇ in UBIAD1 expression-vector-transfected MG-63 cells. MK-4-d₇ levels were correlated with UBIAD1 messenger RNA levels and UBIAD1 protein expression levels.

To confirm that we had identified the *UBIAD1* gene as relating to a biosynthetic enzyme for MK-4, we expressed the most prevalent form of the enzyme in *Spodoptera frugiperda* (Sf9) cells. UBIAD1 expression

was detected in *UBIAD1* baculovirus-infected and not in control-infected Sf9 cells. Sf9 cells showed no measurable conversion activity, but showed conversion activity when infected with *UBIAD1* baculovirus.

LOCALIZATION AND TISSUE EXPRESSION OF UBIAD1

The UBIAD1 enzyme is speculated to be localized in the endoplasmic reticulum (ER) on the basis of its amino-acid structure. To study the subcellular localization of UBIAD1, we generated constructs expressing green fluorescent protein (GFP)-tagged UBIAD1 fusion proteins (UBIAD1-GFP) for the stable transfection of MG-63 cells. The green fluorescence of UBIAD1-GFP decorated the mesh-like structure of the ER in the cytoplasm and co-localized exactly with the red ER marker label (ER-tracker Red) but did not co-localize with the Golgi marker (4,4-difluoro-4-bora-3a,4a-diaza-s-indacene (BODIPY)-TR ceramide). These results strongly indicate that UBIAD1 is a biosynthetic enzyme for MK-4 located in the ER.

We also examined the distributions of *Ubiad1* mRNA in mouse tissues and found that mouse *Ubiad1* mRNA was expressed ubiquitously in all tissues tested. We measured the concentrations of MK-4, MK-4-d₇ and their epoxides in several tissues, including the cerebrum, liver and pancreas of mice orally administered with PK-d₇. MK-4-d₇ biosynthetic activity was correlated with mouse *Ubiad1* mRNA expression in tissues, except for the heart. Further, the tissue accumulation patterns of MK-4-d₇ and its epoxide coincided with those of endogenous MK-4 and its epoxide. The expression of *Ubiad1* mRNA in heart was highest in the tissues tested, whereas the concentrations of MK-4, MK-4-d₇ and their epoxides were relatively low. This may suggest an unknown function of UBIAD1 in addition to MK-4 biosynthesis. Taken together, our results strongly suggest that PK is converted to MK-4 by UBIAD1 and accumulates as MK-4 in tissues.

UBIAD1 HAS THE SIDE-CHAIN CLEAVAGE ACTIVITY AND PRENYLATION ACTIVITY

To ascertain the mechanism for the side-chain cleavage and prenylation activities of UBIAD1, we measured UBIAD1 activity by using the microsomal fraction of UBIAD1 baculovirus-infected Sf9 cells. As the results, we detected the activity of UBIAD1 in converting MK-4-d₇ from PK-d₇ and K₃-d₈ in the microsomes. Moreover, the prenylation activity of UBIAD1 was the side-chain substrate (geranylgeranyl pyrophosphate, GGPP) dose-dependent manner. These results suggest that UBIAD1 is a novel biosynthetic enzyme for MK-4 that may have both side-chain cleavage and prenylation activities.

CONCLUSIONS

Vitamin K is an important factor in proper blood clotting and bone metabolism. MK-4 is not a main bacterial product. MK-4 has a highly specific tissue distribution suggestive of local synthesis from PK, the primary dietary form of vitamin K. However, human MK-4 biosynthesis enzyme has never studied so far. We identified UBIAD1 as a key enzyme for human MK-4 biosynthesis. The function of UBIAD1 is unknown though it has been reported as the candidate gene of Schnyder crystalline corneal dystrophy (SCD), an autosomal dominant disorder involving deposition of cholesterol in the corneal stroma⁽²²⁾. Our finding offer conclusive proof that the biosynthesized MK-4 by UBIAD1 is an important factor with the function as the hormone for anticoagulant therapy, bone health and SCD therapy.

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