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Comparative uptake, metabolism, and utilization of menaquinone-4 and phylloquinone in human cultured cell lines

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Abstract—It is generally accepted that the availability of vitamin K in vivo depends on its homologues, the biological activities of which would differ among organs. To test this hypothesis, we examined the uptake, metabolism, and utilization of menaquinone-4 (MK-4) and phylloquinone (PK) using ^{18}O-labeled compounds in two cultured human cell lines (HepG2 and MG-63). Lipid extracts were prepared from the cells and media after 1, 3, and 6 h of incubation. The detection of the vitamin K analogues (^{18}O-, ^{16}O-quinone, and epoxide forms) was carried out with LC-APCI-MS/MS as previously reported. The ^{18}O of vitamin K was replaced with atmospheric $^{16}O_2$ during the formation of vitamin K epoxide with a carboxylative catalytic reaction. As a result, a significant difference was observed between MK-4 and PK in the amounts taken up into the cells. The ^{18}O-labeled MK-4 was rapidly and remarkably well absorbed into the cells and metabolized to the epoxide form via a hydroquinone form as compared to the ^{18}O-labeled PK. The difference in uptake of MK-4 and PK was not affected by treatment with warfarin although the metabolism of both compounds was markedly inhibited. This methodology should be utilized to clarify some of the actions of vitamin K in target cells and facilitate the development of new vitamin K drugs.

1. Introduction

Vitamin K is a cofactor for γ-glutamyl carboxylase, an enzyme catalyzing the post-transcriptional modification of glutamyl residues to γ-carboxyglutamyl (Gla) residues in some proteins.[1,2] Apart from the classical vitamin K-dependent coagulation proteins synthesized in liver, other Gla proteins such as osteocalcin and matrix-Gla protein (MGP) occur in bone matrix. Osteocalcin, which has three Gla residues, is thought to be involved with the regulation of mineralization.[3,4] In the catalytic reaction of the γ-carboxylation, the reaction cycle of vitamin K is called " the vitamin K cycle" as shown in Figure 1. Vitamin K is first converted to the reduced form, vitamin K hydroquinone, which is required as a cofactor for a microsomal enzyme in cells. During the carboxylation reaction, vitamin K hydroquinone is converted into vitamin K epoxide, and one or two dithiol-dependent reductases mediate the efficient recycling of the epoxide formed. The dithiol-dependent pathway is inhibited by coumarin derivatives such as warfarin.

Regarding chemical structure, vitamin K is a group name for a number of related compounds, which all have a methylated naphthoquinone ring structure, but differ in the length and degree of saturation of their aliphatic side chain at the 3-position. There are two naturally occurring forms of vitamin K: vitamin K_1 (phylloquinone: PK) and vitamin K_2 (menaquinone: MK) (Fig. 2). PK is synthesized by plants and is present in large amounts in various green vegetables, which are the major source of dietary vitamin K. MK has a variable side-chain length of four to thirteen isoprene units and is referred to as MK-*n*, where *n* denotes the number of isoprenoid residues. MK-*n* are bacterial products that are found in fermented foods, as well as in intestinal flora in the colon. Among the MK-*n* homologues, MK-4 is the most potent cofactor of γ-carboxylase. In bone, γ-carboxylated osteocalcin is known to be involved in the bone remodeling system, and pharmacological activ-

Keywords: Vitamin K cycle; Menaquinone-4; Phylloquinone; Uptake; Metabolism; LC-APCI-MS/MS.
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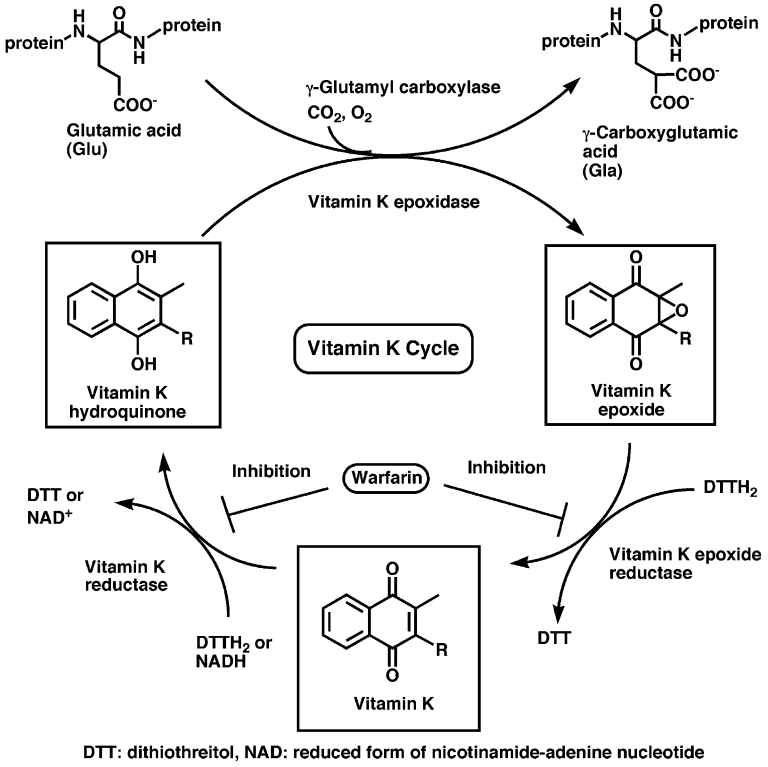
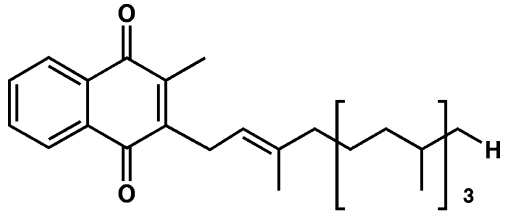
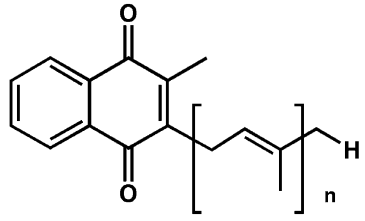


Figure 1. Utilization of vitamin K homologues in the vitamin K cycle: vitamin K in mammals acts as a cofactor in unique post-translational modifications during protein biosynthesis. In this reaction, specific glutamate residues (Glu) in the poly-peptide chain are converted into γ-carboxyglutamate (Gla) by an enzyme known as γ-glutamate carboxylase. Vitamin K quinone is reduced into its hydroquinone form. Oxidation of vitamin K hydroquinone provides the energy for the abstraction of a protein from the γ-carbon in the glutamate residue to generate a carbanion that undergoes carboxylation resulting in a Gla-residue and vitamin K epoxide. The latter can be re-converted into vitamin K quinone by vitamin K epoxide reductase. In this way, vitamin K is recycled several thousand fold.



Vitamin K_1 (Phylloquinone)



Vitamin K_2 (Menaquinone-n)

Figure 2. Structure of vitamin K homologues: There are two types of vitamin K homologues: Vitamin K_1 (phylloquinone, PK) and vitamin K_2 (menaquinone, MK). Vitamin K_1 is a single chemical compound, whereas vitamin K_2 is a general term covering a series of vitamins with a variety of numbers of isoprene units (from 1 to 14) at the 3-position of the quinone structure.

ity of MK-4 has been demonstrated in human osteoblasts in vitro and also in ovariectomized rats, the latter serving as an animal model for osteoporosis.

Two vitamin K homologues, PK and MK-4, are used as bone-building drug in clinical practice. PK is mainly used in Europe and the Unites States, while MK-4 is now available for the treatment of osteoporosis in Japan. Though PK and MK-4 both act as coenzymes for γ-carboxylase in the body, they differ in biological activity. For example, MK-4 was reported to show anti-arteriosclerotic activity[5] and anti-tumor effects in various cancer cells,[6–8] furthermore, it functions as a SXR-specific ligand[9] and the intracellular MK-4-binding protein concerning estradiol was identified.[10] PK, however, has no such activity. It is also revealed that the uptake of these vitamin K homologues into cells depends on the kind of plasma lipoproteins.

The difference in biological activity between PK and MK-4 might depend on uptake, metabolism, and utilization in target cells, therefore, we analyzed the metabolites in the vitamin K cycle using ^{18}O-labeled vitamin K analogues[11] (^{18}O-labeled MK-4 (MK-4-^{18}O) and ^{18}O-labeled PK (PK-^{18}O)) as shown in Figure 3. Recently, Naganathan et al. proposed that central to the oxygenation of vitamin K hydroquinone is the formation of a dioxetane intermediate, followed by the opening of the ring and formation of a strong base. Finally, the molecular oxygen of vitamin K is replaced with atmospheric oxygen during the formation of vitamin K epoxide via a carboxylative catalytic reaction.[12,13] We used this reaction mechanism to investigate the con-

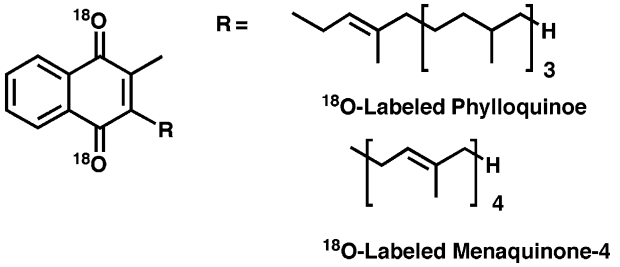


Figure 3. ^{18}O-Labeled vitamin K homologues and their conversion in vitamin K cycle: The intramolecular oxygen of PK and MK-4 was substituted to stable isotope of 18-oxygen. The molecular oxygen of them was replaced with atmospheric $^{16}O_2$ during the formation of vitamin K epoxide to give 'natural' ^{16}O-vitamin K analogues.

version of vitamin K analogues in cells. In short, the molecular oxygen of ^{18}O-labeled vitamin K analogues was replaced with atmospheric $^{16}O_2$ during the formation of vitamin K epoxide to give 'natural' vitamin K analogues. From the results, a significant difference should exist between PK and MK-4 in the amount taken up, metabolized, and utilized in cells.

In this study, we developed a method of simultaneously assaying vitamin K homologues (PK and MK-4) and their metabolites in human cell lines using high-performance liquid chromatography/atmospheric pressure chemical ionization tandem mass spectrometry (LC-APCI-MS/MS). The method was used to determine the metabolites in human cell lines following incubation with vitamin K homologues. An investigation of the comparative uptake, metabolism, and utilization of MK-4 and PK in cultured human cells is described for the first time.

2. Results and discussion

2.1. MS/MS conditions for detection of vitamin K metabolites

We decided to use multiple reaction monitoring (MRM) for the determination of these compounds to achieve high selectivity and sensitivity using the LC-APCI-MS/MS system. First, we attempted to raise the intensity of the protonated molecular ions of vitamin K homologues and their metabolites. As shown in Table 1, a quantitative analysis was carried out using MS/MS-MRM of the precursor ions of vitamin K homologues (*m*/*z* 445 (MK-4), 449 (MK-4-^{18}O), 461 (MK-4 epoxide), 451 (PK), 455 (PK-^{18}O), 467 (PK epoxide)) and the product ions (*m*/*z* 187 (natural vitamin K analogues), *m*/*z* 191 (^{18}O-labeled vitamin K analogues), and *m*/*z* 161 (vitamin K epoxide)) with a dwell time of 500 ms. The effects of the collision energy (Table 1) and collision gas pressure on the intensity were investigated in order to obtain a maximum response of the productions.

Table 1. LC-APCI-MS/MS parameters

Compound	Precursor ion (*m*/*z*)	Collision energy (eV)	Product ion (*m*/*z*)	Retention time (min)
PK-^{18}O	455.4	35	191.3	17.1
PK epoxide	467.4	19	161.2	13.0
PK	451.3	33	187.1	17.1
MK-4-^{18}O	449.3	29	191.2	10.9
MK-4 epoxide	461.3	31	161.0	8.3
MK-4	445.4	31	187.2	10.9
MK-5	531.4	37	187.4	16.2

2.2. Calibration curves

Figure 4A presents typical LC-APCI-MS/MS chromatograms for vitamin ^{18}O-labeled vitamin K (MK-4-^{18}O and PK-^{18}O), vitamin K (MK-4 and PK), vitamin K epoxide (MK-4 epoxide and PK epoxide), and internal standard (MK-5) obtained from the calibration samples. It should be noted that vitamin K analogues were sufficiently separated from each other in a short period of time and the respective chromatograms of the compounds did not interfere with each other. No interfering peak appeared at the retention time of the two compounds on the chromatogram of the blank sample. Figure 5 shows typical calibration curves for MK-4 and PK derivatives. Calibration, using internal standardization, was done by linear regression analysis using five different concentrations, 12.5, 25, 50, 100, and 200 ng/mL. The points were given by the calculated peak area ratio

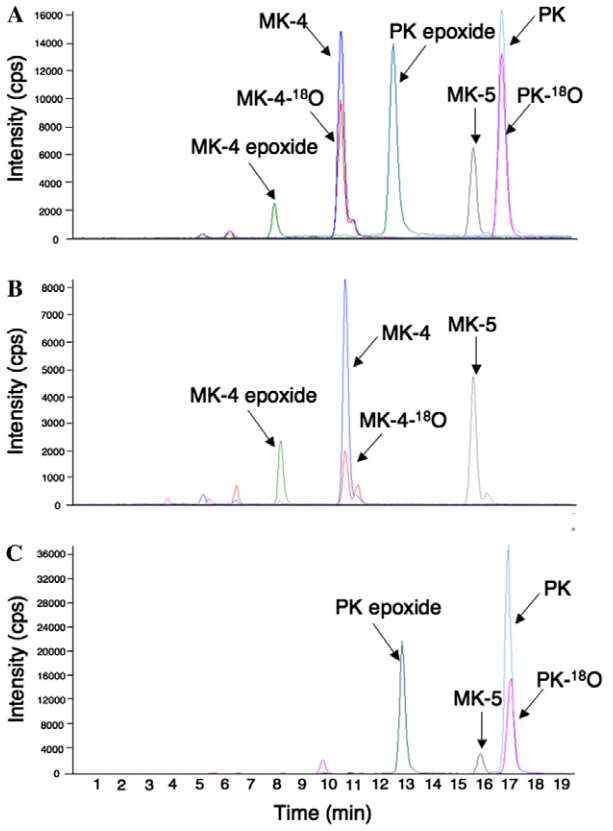


Figure 4. MRM chromatograms: (A) Standard solution (50 ng/mL). The retention times of the standards were as follows: MK-4-^{18}O, 10.9 min; MK-4 epoxide, 8.3 min; MK-4, 10.9 min; PK-^{18}O, 17.1 min; PK epoxide, 13.0 min; PK, 17.1 min; MK-5, 16.2 min. (B) MRM chromatogram for MK-4 derivatives to HepG2 cells (at 1 h). (C) MRM chromatogram for PK derivatives to MG-63 cells (at 6 h).

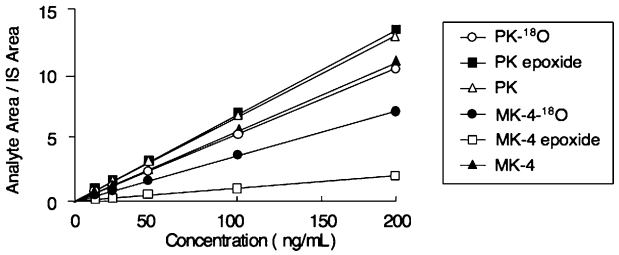


Figure 5. Calibration curves for PK and MK-4 derivatives: PK-^{18}O, $y = 0.0513x$ ($r = 0.9999$); PK epoxide, $y = 0.0685x$ ($r = 0.9999$); PK, $y = 0.0668x$ ($r = 0.9995$); MK-4-^{18}O, $y = 0.035x$ ($r = 0.9998$); MK-4 epoxide, $y = 0.0083x$ ($r = 0.9996$); MK-4, $y = 0.0541x$ ($r = 0.9999$).

of standard and internal standard. Responses in each analysis were linear over the range from 0 to 200 ng/mL for vitamin K derivatives ($r > 0.995$), indicating satisfactory concentration ranges with which to determine the compounds in biological samples.

2.3. Measurement of metabolites of ^{18}O-labeled vitamin K in human cell lines

Figures 4B and C show typical MRM chromatograms of samples derived from HepG2 and MG-63 cells, 1–6 h after the addition of PK-^{18}O and MK-4-^{18}O, respectively. The method has been used to determine ^{18}O-labeled vitamin K (PK-^{18}O and MK-4-^{18}O), vitamin K (PK and MK-4), and vitamin K epoxide (PK epoxide and MK-4 epoxide) in human cultured cell lines in order to investigate metabolism. Those vitamin K analogues were clearly separated the same as standard samples. The concentrations for all of the control samples, to which no vitamin K was added before incubation, were below the quantification limit for both compounds, confirming that endogenous compounds do not interfere with the measurement of ^{18}O-labeled vitamin K, vitamin K epoxide, and vitamin K in human cell lines.

2.4. Study of comparative uptake, metabolism, and utilization of vitamin K homologues in human cultured cell lines

Figures 6 and 7 show the time course of the change on the addition of the stable isotope MK-4-^{18}O or PK-^{18}O to HepG2 and MG-63 cells. The column graph and values on the left indicate the amount of ^{18}O-labeled compounds taken into cells, and of epoxide and natural vitamin K homologues converted in the vitamin K cycle. The results reveal that the ^{18}O-labeled vitamin K absorbed in cells was immediately converted to epoxide and then reduced to natural MK-4. Regarding the concentration of vitamin K homologues in HepG2 cells, the total concentration of vitamin K metabolites gradually increased during 6 h. In MG-63 cells, the metabolic disposition was similar to that in HepG2 cells. But the total amount of vitamin K analogues in MG-63 was smaller than that in HepG2. This finding showed that MK-4

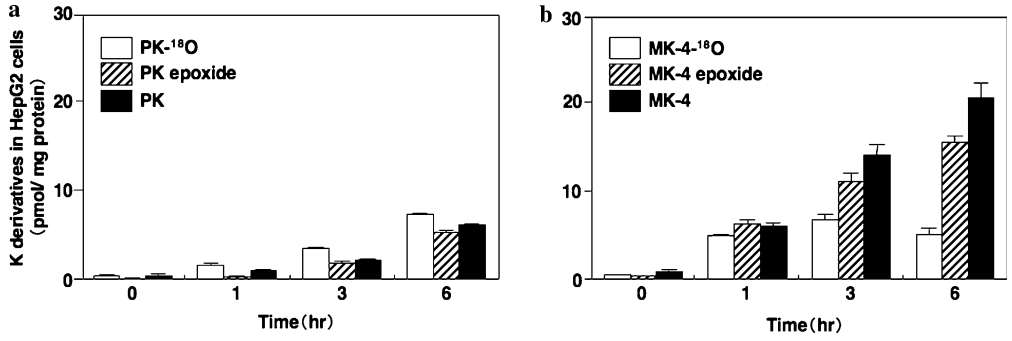


Figure 6. PK and MK-4 derivatives in cells 0–6 h after addition of ^{18}O-labeled vitamin K homologues to HepG2 cells: 220 nM PK-^{18}O or MK-4-^{18}O was added to HepG2 cells. Graphs (a) and (b) represent amount of PK or MK-4 derivatives. The column graph and values on the left indicate the amount of ^{18}O-labeled vitamin K, epoxide, and 'natural' vitamin K in cells.

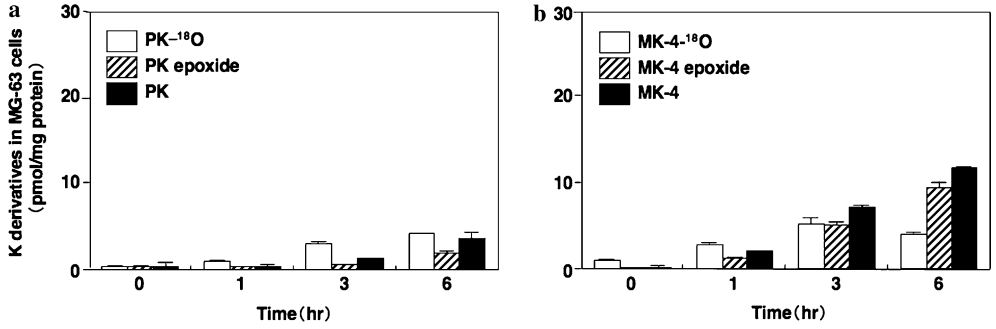


Figure 7. PK and MK-4 derivatives in cells 0–6 h after addition of ^{18}O-labeled vitamin K homologues to MG-63 cells: 220 nM of PK-^{18}O or MK-4-^{18}O was added to MG-63 cells. Graphs (a) and (b) represent amount of PK or MK-4 derivatives. The column graph and values on the left indicate the amount of ^{18}O-labeled vitamin K, epoxide, and "natural" vitamin K in cells.

was taken up and metabolized much faster than PK, and metabolites of PK tend to be accumulated in the cell. In addition, the metabolic rate differed between HepG2 and MG-63. Recently, Sato et al. reported that the turnover of vitamin K is slower in femur than in liver in vivo, and a larger amount of vitamin K is required for its accumulation in the femur than in the liver.[14] Our study in vitro supported this finding, and showed that the metabolic turnover of the vitamin K cycle can be analyzed by measuring metabolites converted from ^{18}O-labeled analogues.

We elucidated the time course of change on the addition of ^{18}O-labeled homologues and warfarin to HepG2 cells. Warfarin, well known as a reductase inhibitor, acts to accumulate epoxide analogues in cells[15] as shown in Figure 8. According to this study, the production of natural ^{16}O-vitamin K was strongly inhibited with both homologues. Therefore, we could clearly observe the metabolic disposition of warfarin activity in the cell.

Concerning the difference in uptake between PK and MK-4, some groups reported that the distribution depends on the lipoproteins in serum.[16–18] In short, PK is predominantly carried in the triacylglycerol-rich lipoprotein fraction, while the uptake of MK-4 related to various lipoproteins. Our findings suggested that the difference is ascribable to lipoprotein in the medium, or other factors might be involved in cells. Furthermore, MK-4 might be utilized in another metabolic pathway different from the vitamin K cycle. One of the main metabolic pathways for absorbed MK-4 is thought to be initial ω-oxidation to ω-COOH, and subsequent β-oxidation to K acid I and K acid II as demonstrated in rats.[19,20] In addition, it was recently shown that MK-4 is able to activate SXR (PXR) and induced expression of cytochrome P450 enzyme.[21] Therefore, regarding the degradation of vitamin K analogues in the cells in our study, one possibility is that MK-4 promotes the expression of CYP3A in cells which is converted to ω-oxidation products as well as other metabolites. We consider that pharmacologically active metabolites might be contained in them, though those studies have not been elucidated so far.

Several articles have reported that MK-4 has various functions other than as a cofactor of γ-carboxylase, such as acting as a SXR ligand, and the intracellular MK-4-binding protein for estradiol was identified. Many of the functions of vitamin K are still unknown. Our methodology would be useful for clarifying some aspects of vitamin K action in target cells and developing new vitamin K drugs.

3. Conclusions

In conclusion, we have demonstrated using LC-APCI-MS/MS method that ^{18}O-labeled vitamin K is absorbed in cells and metabolized to vitamin K epoxide and 'natural' vitamin K in bone as well as in liver. This is the first report on the difference in uptake, metabolism, and utilization between PK and MK-4 in vitro. Our unique assay method will contribute to clarifying the pharmacological mechanism of action of vitamin K analogues in the human body.

4. Experimental

4.1. Reagents and chemicals

HPLC-grade solvents and reagents for chemical synthesis were purchased from Nacalai Tesque, Inc. (Kyoto, Japan) and Wako Pure Chemical Industries, Ltd (Osaka, Japan). The ^{18}O-labeled vitamin K analogues (PK-^{18}O and MK-4-^{18}O) and vitamin K epoxide compounds were synthesized in our laboratory. PK, MK-4, and MK-5 were supplied by Eisai (Japan). The vitamins were solubilized in ethanol. All other chemicals were of analytical grade or better.

4.2. Chemical synthesis and data analysis

The syntheses of ^{18}O-labeled vitamin K analogues and their epoxide compounds were carried out as reported previously and confirmed using NMR spectrometry and high-resolution mass spectroscopy.[11,22]

4.3. Cells and cell culture

The human hepatoblastoma cell line (HepG2) and human osteosarcoma cell line (MG-63) were obtained from Riken cell bank (Tsukuba Science City, Japan). Mini-

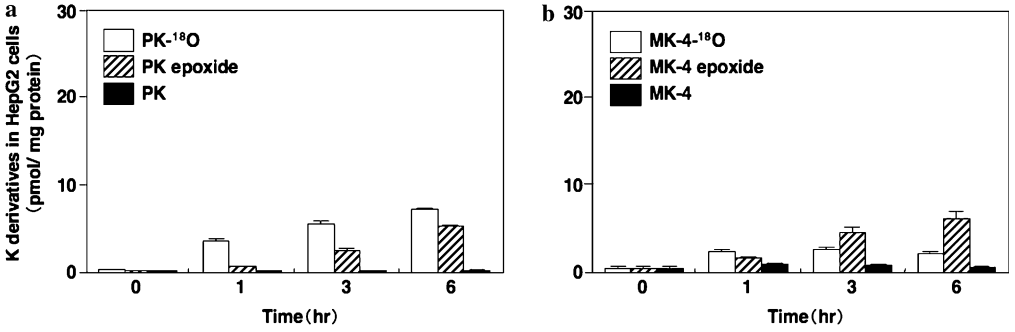


Figure 8. PK and MK-4 derivatives in cells 0–6 h after addition of ^{18}O-labeled vitamin K homologues with warfarin to HepG2 cells: 220 nM PK-^{18}O or MK-4-^{18}O and 25 μM of warfarin were added to HepG2 cells. Graphs (a) and (b) represent amount of PK or MK-4 derivatives. The column graph and values on the left indicate the amount of ^{18}O-labeled vitamin K, epoxide, and "natural" vitamin K in cells.

mum essential medium (MEM), Dulbecco's modified Eagle's medium (DMEM) (low-glucose type), Dulbecco's phosphate-buffered saline (PBS), penicillin–streptomycin (10,000 U/mL penicillin), sodium pyruvate (100 mM), and MEM non-essential amino acid solution (10 mM) were all purchased from Gibco (Grand Island, NY, USA). Trypsin (2.5% solution) and trypsin–EDTA (2.5% trypsin, 1 mM EDTA-solution) were purchased from Nacalai Tesque (Kyoto, Japan). Fetal bovine serum (FBS) was purchased from Termo Trace (Melbourne, Australia). HepG2 was maintained in MEM supplemented with 1 mM sodium pyruvate, 100 μM MEM non-essential amino acid solution, 10% heat-inactivated FBS, and 1% penicillin–streptomycin. MG-63 was maintained in DMEM supplemented with 10% heat-inactivated FBS and 1% penicillin–streptomycin. Cells were cultured in T-75 flasks (BD Falcon, MA, USA) at 37 °C in a humidified 5% CO_2 atmosphere.

4.4. Incubation of cultured human cell lines with ^{18}O-labeled vitamin K homologues

To compare the uptake, metabolism, and utilization of vitamin K_1 and vitamin K_2 in cultured human cell lines, concentrations of vitamin K metabolites in cells were measured after the addition of the vitamins. The experimental medium consisted of culture medium containing MK-4-^{18}O or PK-^{18}O (220 nM) with or without warfarin (25 μM). In the case of HepG2 cells, 8×10^5 cells/well were seeded onto 6-well plates on the first day, and the culture medium was changed every 2 days. Six days later, when cells were confluent, the medium was replaced with the experimental medium. In the case of MG-63 cells, 4×10^5 cells/well were seeded onto 6-well plates on the first day. When cells were confluent 3 days later, the medium was changed to the experimental medium. After cells were incubated in the presence of ^{18}O-labeled vitamin K analogues, they were collected at 0, 1, 3, and 6 h. Cells were washed with cold PBS 3 times and then refrigerated at −30 °C. After being warmed to room temperature, cells were lysed in 1 mL PBS. In this procedure, 10 μL of cell lysate was analyzed for protein determination with a BCA protein assay kit (Pierce, IL, USA).

4.5. Preparation of standard solutions

A stock solution (100 μg/mL) of each standard (PK-^{18}O, MK-4-^{18}O, PK, MK-4, PK epoxide, and MK-4 epoxide) as a reference was prepared in ethanol according to the solubility of the solute and stored in the dark at −30 °C prior to use. For the analytical curves, working solutions of the standard mixture, ranging from 25 to 400 ng/mL, were prepared by dilution of the stock solution with ethanol. The stock solution (10 μg/mL) for the internal standard (MK-5) was prepared by dilution in ethanol and stored in the dark at −30 °C prior to use. Dilution of this solution with ethanol gave working internal standard solutions of 3.6 and 100 ng/mL, respectively. The 3.6 ng/mL mixture was used for the determination of vitamin K homologues in cells and medium. An equal amount of the standard solution (25–400 ng/mL) and internal standard solution (100 ng/mL) gave the solution used for the standard curve. The final concentration ranged from 12.5 to 200 ng/mL in the case of the standard and contained 50 ng/mL of internal standard.

4.6. Sample preparation (extraction of vitamin K and its metabolites from cells)

The medium (1 mL) or cell lysate (PBS solution) (1 mL) in a brown screw-capped Pyrex tube was supplemented with 3.6 ng (in 1 mL of ethanol) MK-5 as internal standard. The addition of extra ethanol (1 mL) to denature the protein and 3 mL of hexane was followed by shaking for 5 min. The solution was centrifuged at 3000 rpm for 5 min, and the upper layer was separated. After the organic layer was evaporated under reduced pressure, the dried sample was dissolved in 60 μL ethanol and sonicated for 10 s. The solutions were transferred to microvials, capped, and placed in a SIL-10AD vp autosampler rack. Aliquots (30 μL) were automatically injected into the HPLC system.

4.7. Apparatus and HPLC conditions

The HPLC analyses were conducted with a Shimadzu HPLC system (Shimadzu, Kyoto, Japan) consisting of a binary pump (LC-10AD liquid chromatography), automatic solvent degasser (DGU-14A degasser), and autosampler (SIL-10AD autoinjector). Separations were carried out using a reversed-phase C18 analytical column (COSMOSIL 5C18-AR-II, 4.6 mm i.d. × 150 mm) (Nacalai Tesque, Kyoto, Japan) with a solvent system consisting of a solvent A and ethanol with a linear gradient from 0 to 30% ethanol. Solvent A contained methanol/0.1% acetic acid aqueous (95:5, v/v). This mobile phase was passed through the column at 1.0 mL/min. The column was maintained at 40 °C with a column oven (CTO-10AC column oven). The HPLC system was controlled by a SCL-10A system controller (Shimadzu, Kyoto, Japan). Acetic acid both functioned as an ion pair reagent during reversed-phase HPLC and facilitated formation of protonated vitamin K, $[M+H]^+$, in the positive ion mode with an APCI. The autosampler was maintained at 25 °C.

4.8. Apparatus and mass spectrometry

Mass spectrometry was performed with an API3000 LC-MS/MS System (Applied Biosystems, CA, USA), equipped with an APCI electrospray interface. All MS data were collected in the positive ion mode and the following settings were used: corona discharge needle voltage, 5.5 kV; vaporizer temperature, 400 °C; sheath gas (high-purity nitrogen) pressure, 50 psi. The electron multiplier voltage was set at 850 eV. Identification and quantitation were based on MS/MS-multiple reaction monitoring (MRM).

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