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Vitamin K suppresses the lipopolysaccharide-induced expression of inflammatory cytokines in cultured macrophage-like cells via the inhibition of the activation of nuclear factor κB through the repression of IKKα/β phosphorylation☆

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Abstract

Vitamin K is essential for blood coagulation and bone metabolism in mammals. This vitamin functions as a cofactor in the posttranslational synthesis of γ-carboxyglutamic acid (Gla) from glutamic acid residues. However, other functions of vitamin K have been reported recently. We previously found that vitamin K suppresses the inflammatory reaction induced by lipopolysaccharide (LPS) in rats and human macrophage-like THP-1 cells. In this study, we further investigated the mechanism underlying the anti-inflammatory effect of vitamin K by using cultures of LPS-treated human- and mouse-derived cells. All the vitamin K analogues analyzed in our study exhibited varied levels of anti-inflammatory activity. The isoprenyl side chain structures, except geranylgeraniol, of these analogues did not show such activity; warfarin did not interfere with this activity. The results of our study suggest that the 2-methyl-1,4-naphtoquinone ring structure contributes to express the anti-inflammatory activity, which is independent of the Gla formation activity of vitamin K. Furthermore, menaquinone-4, a form of vitamin K_2, reduced the activation of nuclear factor κB (NFκB) and inhibited the phosphorylation of IKKα/β after treatment of cells with LPS. These results clearly show that the anti-inflammatory activity of vitamin K is mediated via the inactivation of the NFκB signaling pathway.

Keywords: Anti-inflammation; IKK; Macrophage; Nuclear factor κB; Vitamin K

1. Introduction

It is well known that vitamin K is essential for blood coagulation and also for bone metabolism in mammals. Vitamin K functions as a cofactor of microsomal γ-glutamylcarboxylase (GGCX) in the posttranslational synthesis of γ-carboxyglutamic acid (Gla) from glutamic acid residues of vitamin K-dependent precursor proteins [1,2]. Vitamin K naturally occurs in 2 forms, namely, phylloquinone (vitamin K_1), which is synthesized in plants, and menaquinone (vitamin K_2, or MK-n), which is produced mainly by microorganisms, including intestinal bacteria. Some amount of menaquinone-4 (MK-4), a form of vitamin K_2 containing a geranylgeranyl group (isoprenyl side chain) at the 3-position of 2-methyl-1,4-naphthoquinone, in tissues is derived from diet, especially of animal origin, and some of it is endogenously synthesized from other vitamin K analogues [3–8].

Each vitamin K analogue can serve as a cofactor for Gla formation; however, several recent papers have also reported other novel roles of vitamin K. It has been reported that vitamin K prevents oxidative stress-induced cell death in oligodendrocytes [9]; inhibits the growth of hepatocellular carcinoma cells through the inhibition of nuclear factor κB (NFκB) activation [10]; and stimulates the activity of protein kinase A in neuron-like PC12D cells, hepatocellular carcinoma cells, and osteoblastic cells [11–13]. In addition, MK-4 has several unique functions such as induction of programmed cell death in osteoclasts and several tumor cells in vitro [14–18], inhibition of the differentiation of osteoclasts [19–22] and apoptosis in osteoblasts [23], and direct stimulation of the expression of certain genes via the activation of the nuclear receptor PXR [24–26]. Warfarin, which is an antagonist of Gla formation, does not inhibit the abovementioned novel effects of vitamin K; therefore, these effects of vitamin K are independent of its Gla formation activity.

Previously, we found that the gene expression of acute-phase proteins was markedly enhanced in the liver of vitamin K-deficient rats and that vitamin K supplementation suppressed the lipopolysaccharide (LPS)-induced expression of inflammatory cytokines in rats and human-derived cultured cells [27]. In addition, recent epidemiological studies have revealed that vitamin K status in humans is inversely associated with the levels of circulating inflammatory

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markers [28,29]. These results indicated that vitamin K has an anti-inflammatory effect; however, the detailed mechanism underlying this effect has not yet been fully understood. In this study, we have attempted to clarify the mechanism underlying the anti-inflammatory action of vitamin K by using cultures of human- and mouse-derived cells. We found that vitamin K represses the expression of LPS-induced inflammatory cytokines and inhibits the phosphorylation of IKKα/β, one of the key molecules involved in LPS-induced activation of NFκB. Further, warfarin, a specific inhibitor of Gla formation, did not interfere with this activity. These results indicate that the anti-inflammatory activity of vitamin K involving the inactivation of the NFκB-signaling pathway is independent of its Gla formation activity.

2. Materials and methods

2.1. Materials

Vitamin K_1, menaquinone-3 and warfarin potassium were kindly provided by Eisai (Tokyo, Japan); MK-4 was obtained from Nisshin Pharma (Tokyo, Japan); and menaquinone-7 from J-Oil Mills (Tokyo, Japan). All the vitamin K analogues were dissolved in ethanol at 10 mM. When vitamin K was added in the cell culture medium, the final concentration of ethanol as solvent was adjusted to 0.1%. Warfarin potassium was dissolved in distilled water at a concentration of 10 mM and kept at −20°C before use. Phytol, geraniol, farnesol, and geranylgeraniol were purchased from Sigma (St. Louis, MO, USA), and geranylgeranyl acetone from Eisai.

2.2. Cell culture

Human monocytic THP-1 and mouse RAW264.7 cells were obtained from RIKEN BioResource Center (Tukuba, Japan) and were cultured in RPMI 1640 or Eagle's minimum essential medium (Sigma) supplemented with 10% fetal bovine serum (Biowest, Nuaillé, France), 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C and under 5% CO_2 atmosphere. The THP-1 cells were differentiated for 48 h in the presence of phorbol 12-myristate 13-acetate (10 ng/mL, Wako Pure Chemical Industries, Osaka, Japan) before being used for the actual experiments. Spleen cells were obtained from C57Bl/6Crslc mice purchased from Japan SLC (Shizuoka, Japan) according to the method of Yang et al. [30] and incubated in the RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL of penicillin, and 100 μg/mL streptomycin at 37°C and under 5% CO_2 atmosphere.

2.3. RNA preparation and quantitative reverse transcriptase mediated polymerase chain reaction

The differentiated THP-1 or RAW264.7 cells were incubated in a culture medium in the presence of vitamin K analogues for the indicated time, and then stimulated with LPS (1 μg/mL, *Escherichia coli* serotype O111:B4, L2630; Sigma) for 3 h. The mRNA levels of interleukin-6 (IL-6) in the THP-1 and RAW264.7 cells increased and reached a maximum level at 3 h after LPS stimulation. Therefore, we isolated RNA from these cells 3 h after LPS stimulation. In the case of warfarin administration, cells were treated with both MK-4 and warfarin for 24 h, followed by LPS stimulation. After the treatment, the total RNA was isolated from the cells by using the guanidine-isothiocyanate based reagent Isogen (Nippon Gene, Tokyo, Japan) and used as the template for cDNA synthesis, as described previously [31]. An aliquot of cDNA was used as a template for quantitative polymerase chain reaction (PCR); the Applied Biosystems 7300 Real Time PCR System (Foster City, CA, USA) was used for amplification. The target cDNAs were amplified by the gene-specific primers (Table 1) by using the SYBR Premix Ex Taq solution (Takara Bio, Otsu, Japan). The relative expression level of the mRNAs was normalized according to the obtained amount of eukaryotic translation elongation factor 1α1 (Eef1a1) mRNA.

Table 1
Oligonucleotide sequences that were used as PCR primers

Gene name	Forward primer	Reverse primer
Human interleukin-6	GTGGCTGCAGGACATGACAA	CAATCTGAGGTGCCCATGCTAC
Mouse interleukin-6	AGAGGAGACTTCACAGAGGATACCA	AATCAGAATTGCCATTGCACAAC
Mouse tumor necrosis factor α	GACGTGGAACTGGCAGAAGAG	TCTGGAAGCCCCCCATCT
Mouse interleukin-1β	CTGTGTCTTTCCCGTGGACC	CAGCTCATATGGGTCCGACA
Eef1a1	GATGGCCCCAAATTCTTGAAG	GGACCATGTCAACAATGGCAG

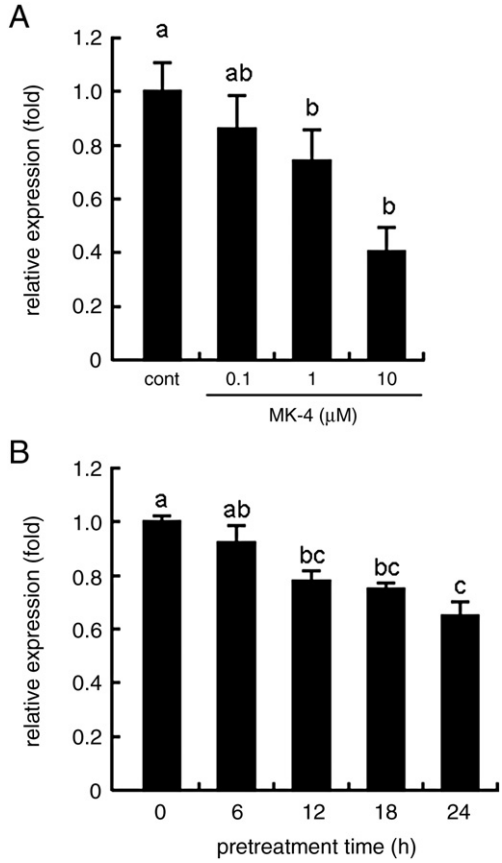

Fig. 1. Menaquinone-4 (MK-4) suppressed the LPS-induced interleukin-6 (IL-6) expression in human THP-1 cells. (A) Human macrophage-like THP-1 cells were pretreated with various concentrations of MK-4 for 24 h, and then treated with LPS (1 μg/mL) for 3 h. The IL-6 mRNA levels were measured by quantitative reverse transcriptase-PCR, normalized to the levels of eukaryotic elongation factor 1α1 (Eef1a1, the internal standard), and expressed as a fold of the control cells values (cont; cells not treated with MK-4). Data are represented as mean±S.E.M., n=6. (B) The macrophage-like THP-1 cells were pretreated with MK-4 (10 μM) for 0, 6, 12, 18, and 24 h, and then treated with LPS (1 μg/mL) for 3 h. The IL-6 mRNA levels were measured by quantitative reverse transcriptase-PCR, normalized to the levels of Eef1a1, and expressed as a fold of the control cells values (pretreatment with MK-4 for 0 h). Data are represented as mean±S.E.M., n=3. The values with different letters (a, b, and c) are significantly different at P<.05.

2.4. Enzyme-linked immunosorbent assay

Differentiated THP-1 cells were incubated with 10 μM MK-4 for 24 h and then stimulated with LPS (1 μg/mL). The culture medium was collected and centrifuged at 800×g for 5 min at 4°C. After separating the supernatant medium, total cell number was determined by counting the cells under the microscope, and WST-1 assay (Takara Bio) was performed to evaluate cell viability. There were no differences in total cell number and cell viability among the different treatment groups. The amount of IL-6 in the obtained supernatant was measured by using the human IL-6 Quantikine ELISA Kit (R & D systems, Minneapolis, MN, USA), and the absorbance was measured using a Spectra Microplate Autoreader (680RX; Bio-Rad, Hercules, CA, USA).

2.5. Reporter gene assay

The reporter plasmids for the luciferase assay, pNFκB-Luc, pAP1-Luc, and pCREB-Luc, were purchased from Clontech Laboratories (Mountain View, CA, USA). The differentiated THP-1 cells (3×10^6 cells per 3-cm culture dish) were cotransfected with 1 μg of either luciferase plasmid and 0.1 μg of β-galactosidase expression plasmid (pmiwZ obtained from JCRB Gene Bank, Tokyo, Japan) by using FuGENE HD (Roche Applied Science, Mannheim, Germany) with or without MK-4 for 24 h, after which they were stimulated with LPS (1 μg/mL) for 4 h. The cells were harvested and lysed using

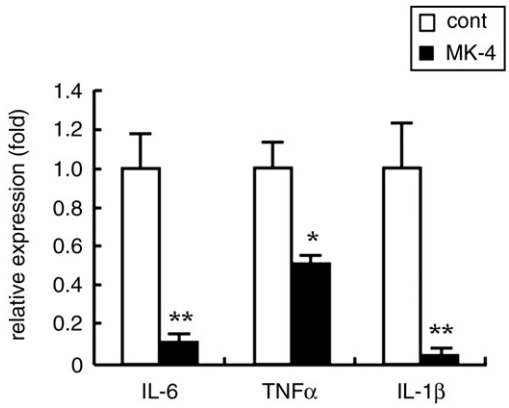

Fig. 2. MK-4 suppressed the mRNA levels of LPS-induced inflammatory cytokines in mouse RAW264.7 cells. RAW264.7 cells were pretreated with MK-4 (10 μM) for 24 h, and then treated with LPS (1 μg/mL) for 3 h. The mRNA levels of IL-6, TNFα, and IL-1β were measured by quantitative reverse transcriptase-PCR, normalized to the levels of Eef1a1 (the internal standard), and expressed as a fold of the control cells values (cont, cells not treated with MK-4). Data are represented as mean±S.E.M., $n=3$. Open bar, control group; closed bar, MK-4 pretreated group. Values are significantly different from those of the control at $^{*}P<.05$ and $^{**}P<.01$.

the Reporter Lysis buffer (Promega, Madison, WI, USA), and the activities of luciferase and β-galactosidase in the lysate were measured by using the Luciferase Assay System (Promega) and Galacto-Light Plus Kit (Tropix, Bedford, MA, USA), respectively.

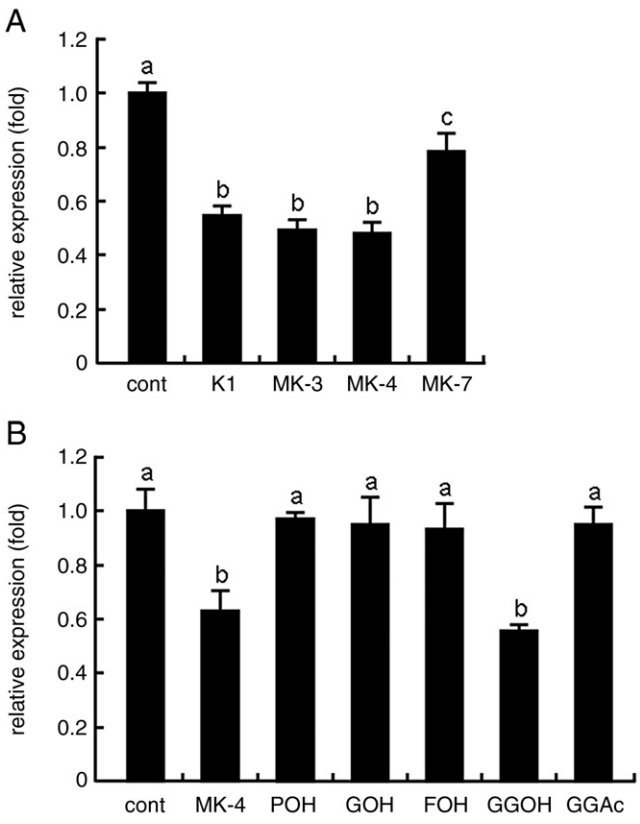

Fig. 3. Vitamin K analogues suppressed the LPS-induced IL-6 expression in human THP-1 cells. Human macrophage-like THP-1 cells were pretreated with various vitamin K analogues (A), various isoprenyl alcohols, or geranylgeranyl acetone (B) (10 μM) for 24 h, and then treated with LPS (1 μg/mL) for 3 h. The IL-6 mRNA levels were measured by quantitative reverse transcriptase-PCR, normalized to the levels of Eef1a1 (the internal standard), and expressed as a fold of the control cells values (cont, no pretreatment). Data are represented as mean±S.E.M., $n=4$–10. Values with different letters (a, b and c) are significantly different at $P<.05$. K1, vitamin K_1; MK-3, menaquinone-3; MK-4, menaquinone-4; MK-7, menaquinone-7; POH, phytol; GOH, geraniol; FOH, farnesol; GGOH, geranylgeraniol; GGAc, geranylgeranyl acetone.

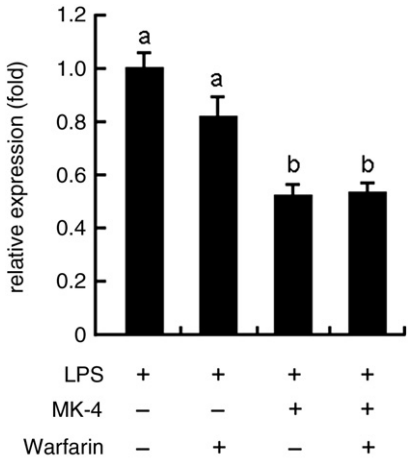

Fig. 4. Warfarin did not affect the decrease in the IL-6 mRNA levels by MK-4 treatment in THP-1 cells. The macrophage-like THP-1 cells were treated with MK-4 (10 μM) and warfarin (10 μM) for 24 h, followed by stimulation with LPS (1 μg/mL) for 3 h. IL-6 mRNA levels were evaluated by quantitative reverse transcriptase-PCR, normalized to the levels of Eef1a1 (the internal standard), and expressed as a fold of control cells values (neither treated with MK-4 nor warfarin). Data are represented as mean±S.E.M., $n=5$. Values with different letters (a and b) are significantly different at $P<.05$.

2.6. Western blot analysis

The differentiated THP-1 cells were incubated with 10 μM MK-4 for 24 h, and then stimulated with LPS (1 μg/mL) for an adequate period. The cells were washed with ice-cold phosphate-buffered saline (PBS) containing 1 mM phenylmethylsulfonyl fluoride, harvested, and lysed with an extraction buffer [10] containing inhibitors for proteinase (Complete proteinase inhibitor cocktail, Roche Applied Science) and phosphatase (PhosSTOP phosphatase inhibitor cocktail, Roche Applied Science). After incubation for 30 min at 4°C, the cell lysate was centrifuged at 15,000×g for 30 min and the supernatant was collected. The protein concentration was determined using a protein assay kit (Bio-Rad). Twenty micrograms of protein was mixed with SDS gel loading buffer and resolved on a 10–20% SDS-polyacrylamide gel electrophoresis (Wako Pure Chemical Industries); subsequently, the proteins were transferred on to a polyvinylidene fluoride membrane (Millipore, Billerica, MA, USA). The membrane was subjected to blocking for 1 h with TBS-T (10 mM Tris-HCl at pH 7.4, 150 mM NaCl, and 0.1% Tween 20) containing 5% bovine serum albumin (Sigma), and then incubated

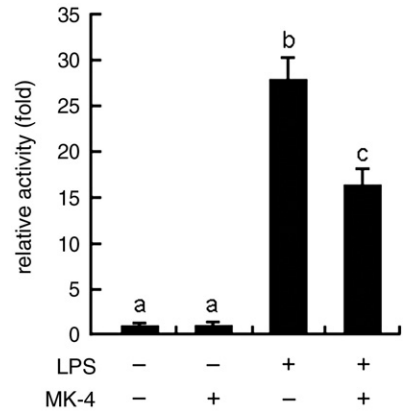

Fig. 5. Pretreatment with MK-4 inhibited the LPS-induced NFκB activation in THP-1 cells. The macrophage-like THP-1 cells were transfected with reporter plasmids driven by NFκB. Twenty-four h after transfection with or without MK-4, cells were treated with LPS (1 μg/mL) for 4 h. Whole cell lysate was prepared and the activity of the reporter gene was measured as described in Materials and methods. Data are represented as mean±S.E.M., $n=3$, normalized to β-galactosidase activity and expressed as a fold of the control cells values (neither treated with LPS nor MK-4). The values with different letters (a, b, and c) are significantly different at $P<.05$.

with antibodies against phosphorylated IRAK1 (Thr209; Abcam, Tokyo, Japan), phosphorylated TAK1 (Thr184/187; Cell Signaling Technology, Danvers, MA, USA), phosphorylated IKKα/β (Ser176/180; Cell Signaling Technology), or phosphorylated p65 (Ser536; Cell Signaling Technology), and detected with the Immobilon Western Detection Reagent (Millipore) using luminescent image analyzer LAS-4000 mini (Fujifilm, Tokyo, Japan). The relative expression level of each protein was normalized according to the amount of α-tubulin detected by its antibody (Sigma).

2.7. Conditioned culture medium

Conditioned culture medium of MK-4 (10 μM)-treated or untreated cells was collected 24 h after the treatment. Both conditioned culture mediums were used to culture another batch of MK-4 untreated cells for 3 h, followed by treatment with LPS for 3 h. The culture medium from cells incubated for 24 h with or without MK-4 were dialyzed against PBS for 24 h at 4°C using a dialysis bag (Sanko Junyaku, Tokyo, Japan) to obtain dialyzed medium and then used to incubate untreated cells in a similar manner.

2.8. Statistical analysis

The results are expressed as mean±S.E.M. The data in Figs. 1A, 1B, 3A, 3B, 4, 5 and 6B were analyzed using one-way analysis of variance and multiple comparisons were made with the Tukey-Kramer test. Data in Figs. 2, 7A, and B were analyzed using Student's *t* test. Statistical analyses were performed using the StatcelQC program (OMS publishing, Saitama, Japan).

3. Results

3.1. Vitamin K suppressed the increased interleukin-6 expression in LPS-treated macrophage-like THP-1 and RAW264.7 cells

We first examined the effect of MK-4 on the LPS-induced IL-6 expression. Differentiation of THP-1 cells into macrophage-like cells was induced by treatment with phorbol ester, and these cells were subsequently treated with LPS (1 μg/mL). At 3 h after LPS treatment, the IL-6 mRNA level in the treated cells was markedly enhanced (by more than eight times) as compared to the level in the LPS-untreated control cells (data not shown). THP-1 cells were pretreated with MK-4 (10 μM) for 24 h, and then treated with LPS for 3 h. The IL-6 mRNA level was noted to be significantly lesser in the MK-4-treated cells than in the control cells (Fig. 1A). This suppressive effect of MK-4 was observed in the cells pretreated with MK-4 for at least 12 h (Fig. 1B). The IL-6 concentration in the culture medium of MK-4-treated cells was also noted to decrease significantly at 12 and 24 h after LPS treatment (data not shown). It is well known that MK-4 induces apoptosis in tumor-derived cells; therefore, we evaluated the viability

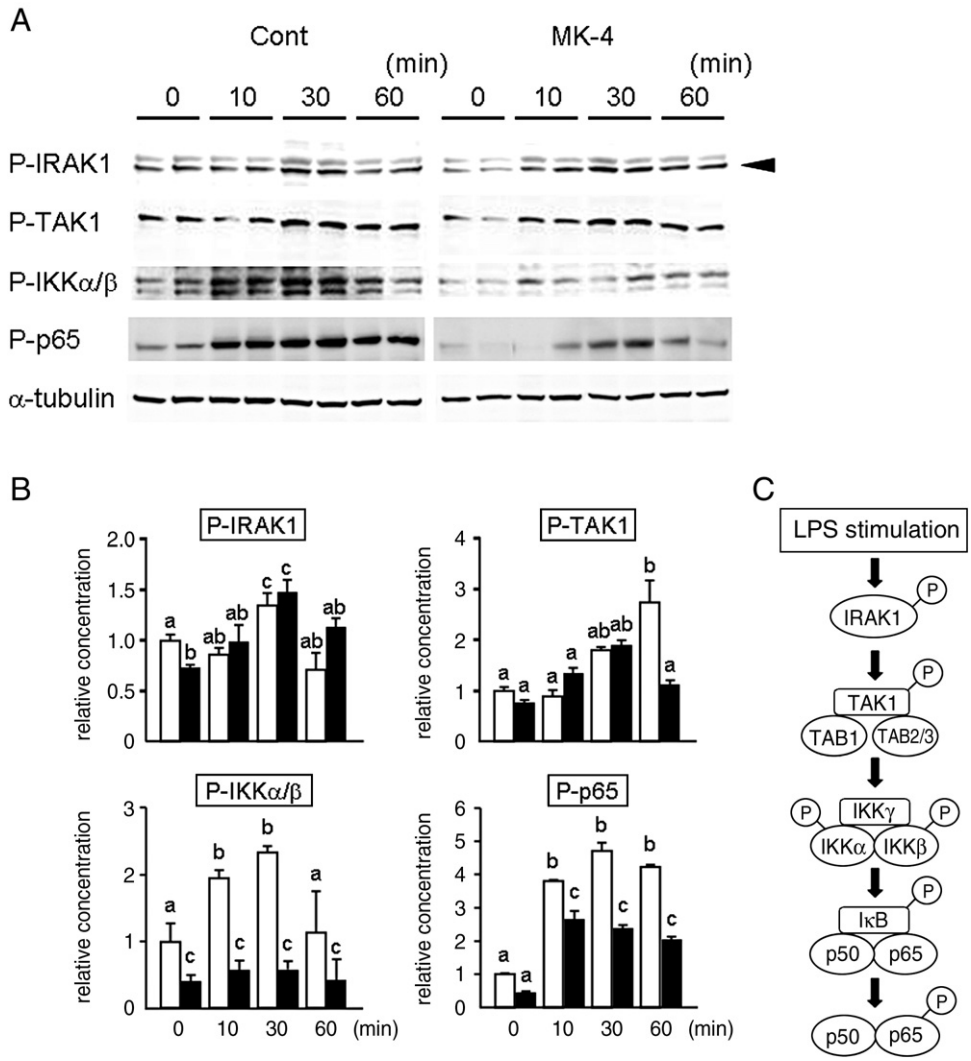

Fig. 6. Pretreatment with MK-4 inhibited the LPS-induced phosphorylation of IKKα/β and NFκB p65 in THP-1 cells. (A) The macrophage-like THP-1 cells were pretreated with MK-4 for 24 h, and then treated with LPS (1 μg/mL). A whole cell extract was prepared at 0, 10, 30, and 60 min after LPS stimulation and the phosphorylation levels of cascade proteins in the NFκB activation pathway were measured by Western blot analysis. Arrowhead, phosphorylated IRAK1. (B) Data are represented as mean±S.E.M., $n=3$, normalized to the α-tubulin levels, and expressed as a fold of the control cells values (cells not treated with MK-4 at 0 min after LPS stimulation). Open bar, no pretreatment with MK-4; closed bar, pretreatment with MK-4. The values with different letters (a, b and c) are significantly different at $P<.05$. (C) Schematic diagram of the LPS-induced activation of NFκB signaling pathway.

of THP-1 cells treated with MK-4 for 24 h by counting the cells under a microscope and by the WST-1 assay. The cell growth and viability of MK-4-treated cells (10 μM) and control cells did not differ significantly (data not shown). Therefore, the decrease in the expression of IL-6 was not caused by the cytotoxic effect of MK-4. Further, we observed that MK-4 treatment reduced the expression of inflammatory cytokine mRNAs (IL-6, TNFα, and IL-1β) in the LPS-treated mouse macrophage-like RAW264.7 cells (Fig. 2) and primary splenic cells (data not shown). These results indicate that pretreatment with MK-4 reduces the LPS-induced expression of inflammatory cytokines and that MK-4 has an anti-inflammatory effect on human and mouse cells.

3.2. 2-Methyl-1,4-naphthoquinone ring structure contributes to express the anti-inflammatory effect

We previously observed that vitamin K_1, another vitamin K analogue, suppressed the LPS-induced IL-6 expression in macrophage-like THP-1 cells [27]. In this study, we examined whether other vitamin K analogues have a similar suppressive effect on the LPS-induced IL-6 expression. As shown in Fig. 3A, not only MK-4, but also vitamin K_1, menaquinone-3, and menaquinone-7 decreased the LPS-induced IL-6 mRNA expression in macrophage-like THP-1 cells. Naturally occurring vitamin K has a phytyl or isoprenyl side chain at 3-position in the naphthoquinone ring. Phytol (side chain structure of vitamin K_1), geraniol, farnesol (side chain structure of menaquinone-3), and geranylgeranyl acetone did not show any suppressive effects on the IL-6 mRNA levels in THP-1 cells (Fig. 3B). However, the geranylgeraniol (side chain structure of MK-4) significantly decreased the IL-6 mRNA level. These results suggest that the anti-inflammatory effect of vitamin K may be attributed to the presence of the naphthoquinone ring.

All vitamin K analogues act as a cofactor for GGCX for the production of Gla-containing proteins. To clarify whether Gla-formation is required to decrease the IL-6 expression, we evaluated the effect of warfarin, which is a γ-glutamylcaboxylation inhibitor. The LPS-induced IL-6 mRNA expression in THP-1 cells was slightly decreased after warfarin treatment; however, warfarin did not interfere with the suppressive effect of MK-4 (Fig. 4). These results suggest that Gla modification is not a pre-requisite for the anti-inflammatory effect of vitamin K.

3.3. MK-4 inhibits the activation of NFκB via LPS stimulation

The expression of *IL-6* gene is tightly regulated by transcription factors, such as activated protein-1 (AP-1), cAMP responsive element binding protein (CREB), and NFκB. MK-4 may inhibit the activity of any of the abovementioned transcription factors leading to a decrease in the LPS-induced IL-6 mRNA expression level. To determine which of the transcription factors is inhibited, we analyzed the activity of transcription factors by using luciferase reporter plasmids with reporter genes containing the corresponding binding nucleotide sequence in their promoter region. Either of the reporter plasmids was transfected into the THP-1 cells; after 24 h, the cells were treated with LPS for 4 h, and their luciferase activities were then measured. It was noted that the treatment of LPS-induced THP-1 cells with MK-4 resulted in a decrease in the luciferase activity of the NFκB reporter gene (Fig. 5), but the activity of AP-1 and CREB reporter genes was unaffected (data not shown). These results indicate that MK-4 inhibits the transcriptional activity of NFκB and reduces the expression of the *IL-6* gene in LPS-treated cells.

Stimulation with LPS triggers a phosphorylation cascade of proteins that results in the activation of NFκB. To determine the molecule that is responsible for the inhibition of NFκB activation by MK-4, we analyzed the phosphorylation status of the proteins in the THP-1 cells after LPS stimulation. Within 30 min after LPS stimulation, the phosphorylation of IRAK1 and TAK1 of the MK-4-treated cells remained unaffected (Figs. 6A and B), while that of IKKα/β and NFκB p65 was significantly reduced. These results indicate that the inhibition of NFκB activity by MK-4 is caused by the inhibition of phosphorylation (i.e., activation) of IKKα/β.

3.4. A conditioned medium of MK-4-treated cells suppresses the LPS-induced IL-6 mRNA expression

Pretreatment with MK-4 is necessary for the suppression of the inflammatory changes in THP-1 cells (Fig. 1B). This observation suggests that MK-4 indirectly inhibits the LPS-induced NFκB activation. MK-4 metabolites or anti-inflammatory substances secreted by the cells after MK-4 treatment are strong candidates for molecules that are responsible for the anti-inflammatory effect. Therefore, we further examined the anti-inflammatory activity of the conditioned medium of MK-4 treated cells. THP-1 cells were treated with MK-4 for 24 h; this conditioned culture medium was then collected and used to culture another batch of THP-1 cells not treated with MK-4. After 3 h of incubation with the conditioned medium, the cells were treated with LPS for 3 h. It was noted that the conditioned medium of MK-4 treated cells significantly decreased the levels of LPS-induced IL-6 mRNA expression in the THP-1 cells (Fig. 7A). A similar finding was observed in the case of RAW264.7 cells (data not shown). When the conditioned medium of MK-4 treated cells was dialyzed against PBS to remove low-molecular-weight components (approximately less than 10,000), such as the metabolites of MK-4, the IL-6 mRNA levels were noted to decrease markedly (Fig. 7B).

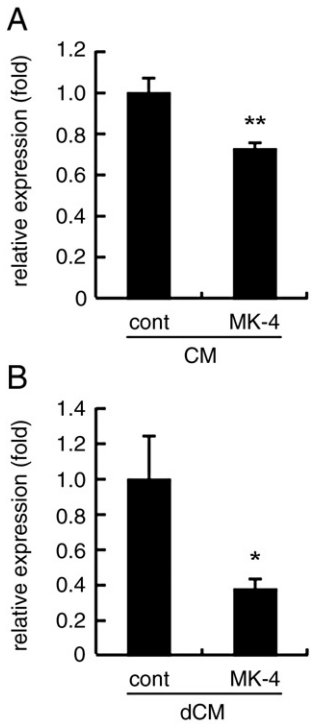

Fig. 7. Conditioned medium of MK-4-treated THP-1 cells suppressed the LPS-induced IL-6 expression in THP-1 cells. The macrophage-like THP-1 cells were pretreated with the conditioned medium (CM) (A) or the dialyzed conditioned medium (dCM) (B) of MK-4 treated (MK-4) or untreated cells (cont) for 3 h, and then treated with LPS for 3 h. The IL-6 mRNA levels were measured by quantitative reverse transcriptase-PCR, normalized to the levels of Eef1a1 (the internal standard), and expressed as a fold of the control cells values (cont). Data are expressed in terms of mean±S.E.M., $n=6$ (A) and 3 (B). The values are significantly different compared with those of control at $^{*}P<.05$ and $^{**}P<.01$.

These results indicate that the anti-inflammatory effect of MK-4 is in fact mediated by the high molecular-weight-substances secreted by MK-4-treated cells.

4. Discussion

We showed that vitamin K repressed the expression of LPS-induced inflammatory cytokines in cultured cells. In human- and mouse-derived macrophage-like cells and primary splenic cells of mouse, MK-4 was found to repress the LPS-induced expression of IL-6. The other vitamin K analogues also showed this effect. Since this effect requires pretreatment of the cells with MK-4 before the LPS treatment, the repression of inflammatory cytokine expression by vitamin K was not observed as a direct action of vitamin K but was possibly mediated by the anti-inflammatory substances generated during the pretreatment period with vitamin K. Vitamin K treatment induced the inhibition of phosphorylation of IKKα/β that is required for the LPS-induced activation of NFκB. These data indicate that vitamin K exerts its anti-inflammatory effect by suppressing the LPS-induced activation of NFκB pathway.

The main physiological role of vitamin K is the posttranslational modification (carboxylation of a Glu residue to generate a Gla residue) of vitamin K-dependent proteins. Vitamin K is converted from the oxidized form (quinone) to the reduced form (hydroquinone) before the reaction of γ-carboxylation of a Glu residue. Vitamin K epoxide reductase (VKOR) catalyzes this reaction. Warfarin, an anticoagulant that prevents thrombosis, acts as a competitive inhibitor of VKOR and inhibits Gla formation. The suppression of the LPS-induced IL-6 expression by MK-4 was not affected by warfarin (Fig. 4). Moreover, the presence of the 2-methyl-1,4-naphthoquinone structure, and not the isoprenyl side chains contributes to express the suppression (Fig. 3B). Therefore, we presumed that the anti-inflammatory effect of vitamin K is a novel property of chemicals that have the naphthoquinone structure, but are not involved in Gla formation in proteins.

The suppression of IL-6 expression by vitamin K has been previously investigated by other researchers. Reddi et al. reported that the LPS-induced IL-6 secretion in human fibroblasts was suppressed by vitamin K [32]. They presumed that the active compound responsible for this suppressive effect was a metabolite of vitamin K because the water-soluble metabolites of vitamin K had the highest activity among the vitamin K analogues that they had examined. In our experiment, the pretreatment of vitamin K was a key step that resulted in the suppression of IL-6 expression; further, the conditioned medium of vitamin K-treated cells also showed the suppressive effect. These data suggest that the metabolites of vitamin K may themselves exert a suppressive effect. However, the dialyzed conditioned medium without water-soluble, low-molecular-weight substances (approximately less than 10,000) also showed a similar activity. Thus, we presume that the suppression of IL-6 expression by vitamin K could be mediated by high-molecular-weight anti-inflammatory substances secreted by the vitamin K-treated cells. Further studies to evaluate the detailed properties of this anti-inflammatory factor are necessary so as to reveal the complete molecular mechanism underlying the anti-inflammatory effect of vitamin K.

Further, we showed that the suppression of IL-6 expression was caused by the inhibition of NFκB activation by the reporter gene system. NFκB is a transcription factor that is closely related to a number of cellular functions; it is not only involved in immune response but also in cell proliferation, differentiation, and anti-apoptotic responses [33]. NFκB interacts with IκB; which is present in an inactive form in the cytoplasm to prevent the nuclear translocation of NFκB. Cytokines and other extracellular stimuli activate IKK, and the resulting phosphorylated IκB is rapidly degraded by the ubiquitin proteasome system (Fig. 6C). The free NFκB translocates to the nucleus and activates the expression of its target genes. We observed that in LPS-treated cells, the phosphorylation of IKKα/β was significantly decreased after MK-4 treatment while the phosphorylation level of TAK1, which phosphorylates IKK, was not affected (Fig. 6B). Ozaki et al. showed that MK-4 inhibited the activation of NFκB stimulated by TNFα, IL-1β, and phorbol ester in human hepatocellular carcinoma cells [10]. Further, they reported that MK-4 inhibited the degradation of IκBα by the repression of IKK activity. The intracellular signals induced by TNFα and IL-1β as well as LPS are integrated by a common mediator TAK1, which regulates IKK activity by its phosphorylation. Thus, when the expression plasmid p65 of NFκB and NFκB reporter plasmid were cotransfected with HepG2 and LS180 (derived from human liver and colon cancers, respectively), the activity of reporter gene was enhanced by p65 overexpression, but MK-4 treatment did not reduce the enhanced activity of the reporter gene (Ohashi, unpublished data). Therefore, we speculate that MK-4 inhibits the activation of NFκB by decreasing the phosphorylation of IKKα/β. One possible mechanism is that MK-4 treatment may enhance the activity of protein phosphatases PP2A and PP2C that may remove phosphates from serine residues in the catalytic domain of IKKα/β [34,35]. Another possibility is that MK-4 treatment may lead to the dissociation of the IKK complex. IKK consists of 3 subunits, α, β and γ. IKKγ functions as the regulatory subunit and recognizes K63-ubiquitinated modification in upstream receptor/adaptor protein platform (e.g., TRAF6 and IRAK1). TAK1 also interacts with same platform via TAB2/3 that interacts with TAK1 and recognizes K63-ubiquitinated proteins. The recruitment of IKK trimer complex and TAK1-TAB2/3 on the platform is necessary to induce the phosphorylation of IKKα/β. The dissociation of IKK complex may reduce the rate of effective phosphorylation of IKKα/β by TAK1. Further experiments should be performed to elucidate the detailed mechanism.

In conclusion, vitamin K exerts its anti-inflammatory effect via the repression of NFκB activation. This effect of vitamin K is different from its well-known function of Gla formation in proteins. A large amount of vitamin K is present in the extra-hepatic or the bone tissues, including the brain and the gonadal tissues, but the biological significance of vitamin K in these tissues remains unclear. Our findings can contribute to further clarification of the role of vitamin K in these tissues. Vitamin K may be involved in diverse cellular functions by the modulation of NFκB activity.

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