Clinical & Experimental Immunology
The Journal of Translational Immunology

Clinical and Experimental Immunology ORIGINAL ARTICLE doi:10.1111/j.1365-2249.2009.04083.x

Vitamin K_3 attenuates lipopolysaccharide-induced acute lung injury through inhibition of nuclear factor-κB activation

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Accepted for publication 19 November 2009
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Summary

Vitamin K is a family of fat-soluble compounds including phylloquinone (vitamin K_1), menaquinone (vitamin K_2) and menadione (vitamin K_3). Recently, it was reported that vitamin K, especially vitamins K_1 and K_2, exerts a variety of biological effects, and these compounds are expected to be candidates for therapeutic agents against various diseases. In this study, we investigated the anti-inflammatory effects of vitamin K_3 in *in vitro* cultured cell experiments and *in vivo* animal experiments. In human embryonic kidney (HEK)293 cells, vitamin K_3 inhibited the tumour necrosis factor (TNF)-α-evoked translocation of nuclear factor (NF)-κB into the nucleus, although vitamins K_1 and K_2 did not. Vitamin K_3 also suppressed the lipopolysaccharide (LPS)-induced nuclear translocation of NF-κB and production of TNF-α in mouse macrophage RAW264·7 cells. Moreover, the addition of vitamin K_3 before and after LPS administration attenuated the severity of lung injury in an animal model of acute lung injury/acute respiratory distress syndrome (ARDS), which occurs in the setting of acute severe illness complicated by systemic inflammation. In the ARDS model, vitamin K_3 also suppressed the LPS-induced increase in the serum TNF-α level and inhibited the LPS-evoked nuclear translocation of NF-κB in lung tissue. Despite marked efforts, little therapeutic progress has been made, and the mortality rate of ARDS remains high. Vitamin K_3 may be an effective therapeutic strategy against acute lung injury including ARDS.

Keywords: ARDS, LPS, NF-κB, TNF-α, vitamin K_3

Introduction

Vitamin K is a family of fat-soluble compounds including phylloquinone (vitamin K_1), menaquinone (vitamin K_2) and menadione (vitamin K_3) (Fig. 1). Vitamin K_1, which is the best-known member of the vitamin K family, is found abundantly in many plants and algae, especially green leafy vegetables. Vitamin K_2 is produced by bacteria. Vitamin K_3, a synthetic analogue of vitamin K, acts as a provitamin that is converted into a vitamin in the body. Vitamin K plays important roles in physiological functions; it acts as a critical factor in blood coagulation and bone metabolism in mammals [1]. Recently, it was reported that vitamin K has anti-cancer activity, and the anti-cancer effects of vitamin K_3 against hepatoma cells was found to be greater than those of vitamins K_1 and K_2 [2]. Vitamin K_3 strongly suppressed the proliferation of human colon cancer cells and induced apoptosis by inhibiting human DNA polymerase γ, which is a mitochondrial DNA polymerase, but vitamins K_1 and K_2 did not [1]. The inhibitory effects of vitamin K_3 on angiogenesis in a rat aortic ring model were significantly stronger than those of vitamins K_1 and K_2 [3]. From these results, vitamin K_3 is a candidate as a therapeutic agent against various diseases.

Acute lung injury/acute respiratory distress syndrome (ARDS) occurs in the setting of acute severe illness complicated by systemic inflammation. ARDS represents a state of excess production of inflammatory mediators from immune cells, such as cytokines, chemokines, adhesion molecules and bioactive lipid products [4]. The most common pathological condition of ARDS is sepsis. Lipopolysaccharide (LPS) released during sepsis is the major stimulus for the release of inflammatory mediators [5]. Administration of LPS to experimental animals causes the pathological condition of ongoing sepsis and concomitant ARDS-like lung injury, including polymorphonuclear neutrophil sequestration and

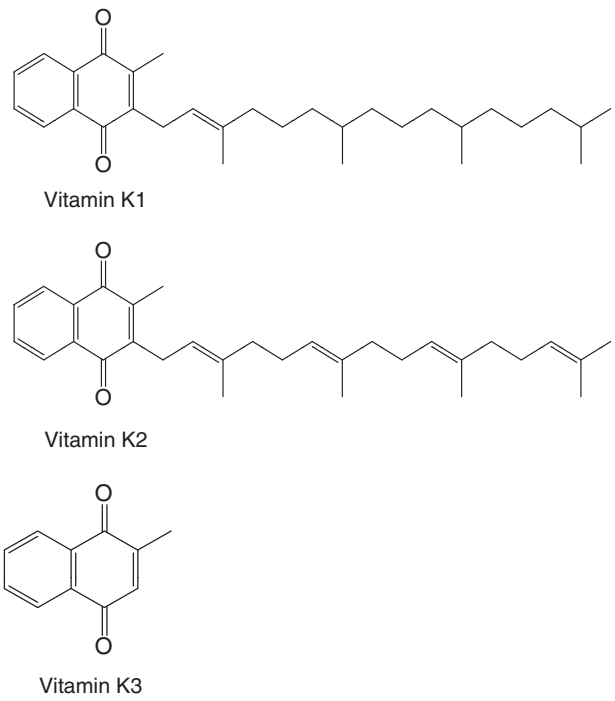

Fig. 1. Chemical structures of vitamins K_1, K_2 and K_3.

lung oedema [6]. Despite marked efforts, little therapeutic progress has been made, and the mortality rate of ARDS remains high [7]. Therefore, the novel effective therapeutic strategy for ARDS is required.

In this study, we examined the suppressive effects of vitamin K_3 on nuclear factor (NF)-κB activation and the following inflammatory responses in *in vitro* experiments using human embryonic kidney (HEK)293 cells and mouse macrophage RAW264·7 cells. Next, we investigated whether vitamin K_3 was able to attenuate the severity of lung injury in an LPS-induced ARDS model.

Methods

Chemicals

Vitamins K_1, K_2 and K_3 and LPS were purchased from Sigma (St Louis, MO, USA). For Western blot analysis and immunofluorescence staining, anti-NF-κB p65 antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and anti-rabbit IgG antibody was from Thermo Scientific (Kanagawa, Japan).

Cell culture

HEK293 cells and murine macrophage cell line RAW264·7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 4·5 g of glucose per litre plus 10% fetal calf serum, 5 mM L-glutamine, 50 units/ml penicillin and 50 units/ml streptomycin and were cultured in a humidified incubator with an atmosphere of 95% air and 5% CO_2 at 37°C.

Preparation of nuclear proteins

HEK293 cells, RAW264·7 cells and peritoneal macrophages on a six-well plate at 5×10^5 cells/well were incubated with 50 μM vitamins K_1, K_2 or K_3 for 30 min followed by treatment with 100 ng/ml tumour necrosis factor (TNF)-α or 100 ng/ml LPS for 30 min. Preparation of nuclear proteins was performed according to the method of a previous report [8]. Briefly, the cells were washed three times with phosphate-buffered saline (PBS) and were collected with hypotonic buffer [10 mM Tris-HCl, pH 7·5, 10 mM KCl, 1·5 mM $MgCl_2$, 0·1% nonidet P-40, 1 mM DTT, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM phenylmethanesulphonylfluoride (PMSF) and 1 mM Na_3VO_4]. The cell lysate was incubated on ice with occasional mixing for 15 min, and the nuclei and the cytosol were separated by centrifugation at 1600 ***g*** for 10 min at 4°C. The nuclei were resuspended in hypertonic buffer [20 mM Tris-HCl, 20% glycerol, 500 mM NaCl, 1·5 mM $MgCl_2$, 0·2 mM ethylenediamine tetraacetic acid (EDTA), 1 mM dithiothreitol (DTT), 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM PMSF and 1 mM Na_3VO_4] and incubated on ice with occasional mixing for 30 min. The nuclear proteins were obtained by centrifugation at 15 000 ***g*** for 20 min at 4°C. The protein concentration was measured using a bicinchoninic (BCA) assay kit, according to the manufacturer's protocol.

Western blot analysis

The nuclear proteins (30–50 μg protein) were boiled in a quarter-volume of sample buffer [1 M Tris-HCl, pH 7·5, 640 mM 2-mercaptoethanol, 0·2% bromphenol blue, 4% sodium dodecyl sulphate (SDS) and 20% glycerol] and were separated on 10% SDS-polyacrylamide gels. The proteins on the gels were transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with 1% skimmed milk in Tris-buffered saline Tween 20 (TBS-T) (10 mM Tris-HCl, pH 8·0. 100 mM NaCl, 0·5% Tween-20) and was probed with anti-NF-κB p65 antibody (1 : 1000) or β-actin antibody (1 : 5000) before being reacted with the corresponding horseradish peroxidase-conjugated secondary antibody (1 : 20 000). The protein/antibody complex was visualized with ChemiLumiONE (Nacalai Tesque, Kobe, Japan) and was detected using an Image Reader (LAS-3000 Imaging System, Fuji Photo Film, Tokyo, Japan).

Immunofluorescence study

HEK293 cells and RAW264·7 cells were incubated with 50 μM vitamin K_3 for 30 min followed by treatment with 100 ng/ml TNF-α or 100 ng/ml LPS for 30 min. The cells were fixed with 4% paraformaldehyde for 10 min and were incubated with 0·2% saponin for 30 min. The cells were

blocked with 10% normal goat serum for 30 min followed by incubation with anti-NF-κBp65 antibody (1:1000) for 60 min and the addition of Alexa546-labelled anti-rabbit IgG antibody (1:400) and Topro in PBS (1:1000). After 60 min, NF-κB p65 was visualized with a fluorescent light microscope (ZEISS, Göttingen, Germany).

DNA binding activity of NF-κBs

HEK293 cells were incubated with 50 μM vitamin K_3 for 30 min followed by treatment with 100 ng/ml TNF-α for 30 min. After stimulation with TNF-α for 30 min, the nuclear proteins were prepared to evaluate the DNA binding activity of NF-κB p50 and p65. Their DNA binding activity was quantified using a commercially available NF-κB (human p50/p65) Combo Transcription Factor Assay Kit (Cayman Chemical Co., Ann Arbour, MI, USA) according to the manufacturer's protocol.

Preparation of peritoneal macrophages

All animal studies were performed according to the Kobe University Animal Experimentation Regulations. Male C57BL/6 mice were injected intraperitoneally with 100 μl of 3% thioglycollate solution. After 7 days, the mice were injected intraperitoneally with PBS, and the peritoneal cavity of the mice was washed with PBS. PBS was collected and peritoneal macrophages were separated from the PBS by centrifugation at 300 ***g*** for 5 min [9].

Measurement of TNF-α level in the cell culture medium

The RAW264·7 cells were seeded on a 12-well plate at 1×10^5 cells/well and incubated for 24 h. The cells were pretreated with vitamin K_3 at various concentrations for 30 min before the addition of 100 ng/ml LPS. After stimulation with LPS for 24 h, the cell culture medium was collected to measure the TNF-α level. The TNF-α concentration was quantified using a commercially available enzyme-linked immunosorbent assay (ELISA) development system (Bay Bioscience Co. Ltd, Kobe, Japan) according to the manufacturer's protocol.

Animal experiments

C57BL/6 mice bred in-house with free access to food and water were used for all experiments. All the mice were kept under 12-h light/dark cycles and housed at 25°C. The animal experiments were designed to minimize animal suffering and to use the minimum number of animals possible to obtain a valid statistical evaluation. Male 8-week-old C57BL/6 mice were injected intraperitoneally with 100 mg/kg body weight (BW) vitamin K_3 or corn oil as a vehicle control. After 30 min, the mice were injected intraperitoneally with 15 mg/kg BW LPS or saline as a vehicle control. After 1 h of 15 mg/kg BW LPS administration, the mice were killed and the blood was collected. The serum was separated from blood by centrifugation at 16 000 ***g*** for 10 min at 4°C, and the TNF-α level in serum was measured using a commercially available ELISA development system (Bay Bioscience Co. Ltd), according to the manufacturer's protocol. Before and after 30 min of 15 mg/kg BW LPS administration, the mice were injected intraperitoneally with100 mg/kg BW vitamin K_3 or corn oil as a vehicle control. After 6 h of 15 mg/kg BW LPS administration, the mice were killed and their caudal lobes were removed and immediately put into 10% neutral formalin solution for fixation. The tissues were embedded in paraffin and cut into 5-μm-thick slices. Conventional haematoxylin and eosin staining (H&E ×400) was performed. Half of the lung was also used as a tissue sample. The tissue samples were homogenized in lysis buffer A (10 mM Tris-HCl, 10 mM KCl, 1·5 mM $MgCl_2$, 0·5% Triton X-100, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM PMSF, 1 mM Na_3VO_4 and 1·0 mM DTT). The cell lysate was incubated on ice for 10 min and centrifuged at 1600 ***g*** for 10 min at 4°C. The precipitation was resuspended in lysis buffer A, incubated on ice with 10 min and centrifuged at 1600 ***g*** for 15 min at 4°C. After this process was repeated three times, the precipitation obtained was suspended in 1 ml of lysis buffer A without Triton X-100. The homogenates were vortexed for 30 s and then centrifuged at 1600 ***g*** for 10 min at 4°C. The precipitation was resuspended in lysis buffer B (10 mM Tris-HCl, 25% glycerol, 420 mM NaCl, 1·5 mM $MgCl_2$, 0·2 mM EDTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM PMSF, 1 mM Na_3VO_4 and 1 mM DTT) and incubated on ice for 30 min with occasional mixing. The homogenates were centrifuged at 15 000 ***g*** for 20 min at 4°C, and the supernatant obtained was used as a source of nuclear proteins for Western blot analysis. Before and after 30 min of 15 mg/kg BW LPS administration, the mice were injected intraperitoneally with100 mg/kg BW vitamin K_3 or corn oil as a vehicle control. After 24 h of 15 mg/kg BW LPS administration, the mice lungs were resected and lavaged. For lavage, the diaphragm was punctured, and the trachea was exposed, nicked and then cannulated with 18-gauge injection needle. The cannula was secured with 3-O surgical silk (Ethicon, Somerville, NJ, USA), and the lungs were infused with 500 μl PBS using a 1-ml syringe. In one set of animals ($n = 3$ per group), the lungs were lavaged three times with 1 ml of PBS. The total cell number was determined using a haemocytometer. The number of neutrophils was determined on bronchoalveolar lavage (BAL) smear slides that were stained with Diff-Quick (Sysmex International Reagents, Kobe, Japan).

Statistical analysis

All data are expressed as means ± standard error (s.e.) of at least three independent determinations for each experiment. Statistical significance was analysed using the Student's *t*-test, and a level of probability of 0·05 was used as the criterion for significance.

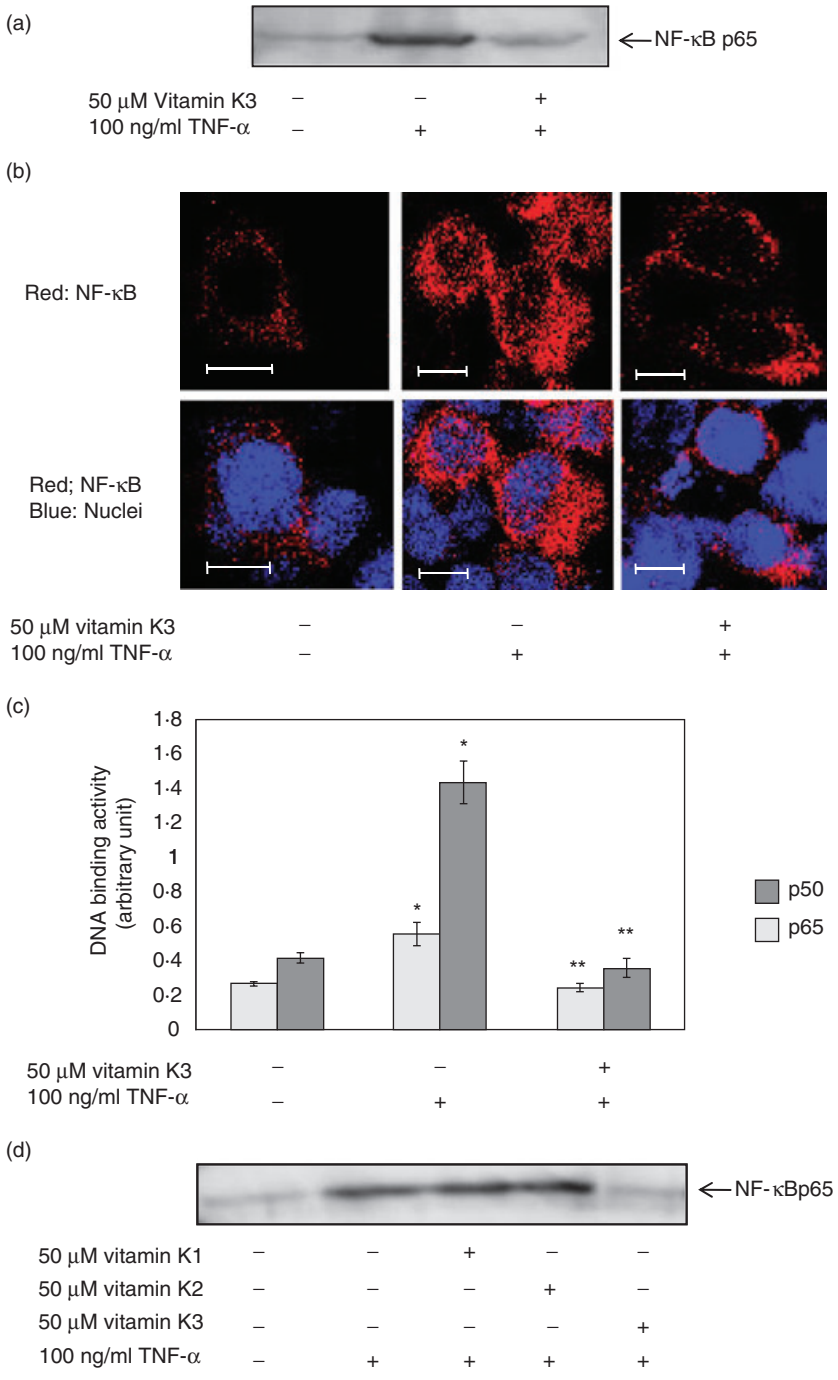

Fig. 2. Inhibitory effects of vitamin K_3 on the tumour necrosis factor (TNF)-α-evoked nuclear factor (NF)-κB activation in human embryonic kidney HEK293 cells. HEK293 cells were incubated with 50 μM vitamin K_3 or dimethylsulphoxide (DMSO) as a vehicle control for 30 min, followed by treatment with 100 ng/ml TNF-α for 30 min. (a) The nuclear proteins were prepared from the cells and were subjected to Western blot analysis for evaluation of the nuclear translocation of NF-κB p65. (b) The cells were fixed in 4% paraformaldehyde, and the nuclear translocation of NF-κB p65 was assessed by immunofluorescence microscopy (red: NF-κB; blue: nuclei). Scale bar, 10 μm. Typical pictures are shown from at least triplicate determinations. (c) The nuclear proteins were prepared from the cells, and the DNA binding activity of NF-κB p50 and p65 were evaluated. Light grey bar: NF-κB p50; dark grey bar: NF-κB p65. Each data value is expressed as mean ± standard error of duplicates of three experiments. The single asterisk indicates the presence of significant differences between negative control and TNF-α alone ($P < 0{\cdot}05$), and the double asterisks indicate the presence of significant differences between TNF-α alone and vitamin K_3 plus TNF-α ($P < 0{\cdot}05$). (d) HEK293 cells were pretreated with 50 μM vitamins K_1, K_2 and K_3 or DMSO as a vehicle control for 30 min followed by the addition of 100 ng/ml TNF-α. After 30 min the nuclear proteins were prepared, and NF-κB p65 in the nucleus was detected by Western blot analysis. Typical pictures are shown from at least triplicate determinations.

Results

Inhibitory effects of vitamin K_3 on the TNF-α-induced activation of NF-κBs in HEK293 cells

First, we investigated whether vitamin K_3 was able to inhibit the nuclear translocation of NF-κB p65 and the binding of NF-κBs to DNA by TNF-α stimulation in HEK293 cells. TNF-α, an inflammatory cytokine, activates the NF-κB signalling pathway via its binding to the TNF-α receptor (TNFR) and initiates inflammatory responses, resulting in various inflammatory diseases [10]. In Western blot analysis (Fig. 2a) and the immunofluorescence study (Fig. 2b), 100 ng/ml TNF-α caused the translocation of NF-κB p65 into the nucleus, and 50 μM vitamin K_3 inhibited the 100 ng/ml TNF-α-caused nuclear translocation. Vitamin K_3 at 50 μM also reduced significantly the DNA binding activity of NF-κB p50 and p65 in HEK293 cells stimulated with 100 ng/ml TNF-α (Fig. 2c). Vitamins K_1 and K_2, which belong to the vitamin K family, were reported to have anti-

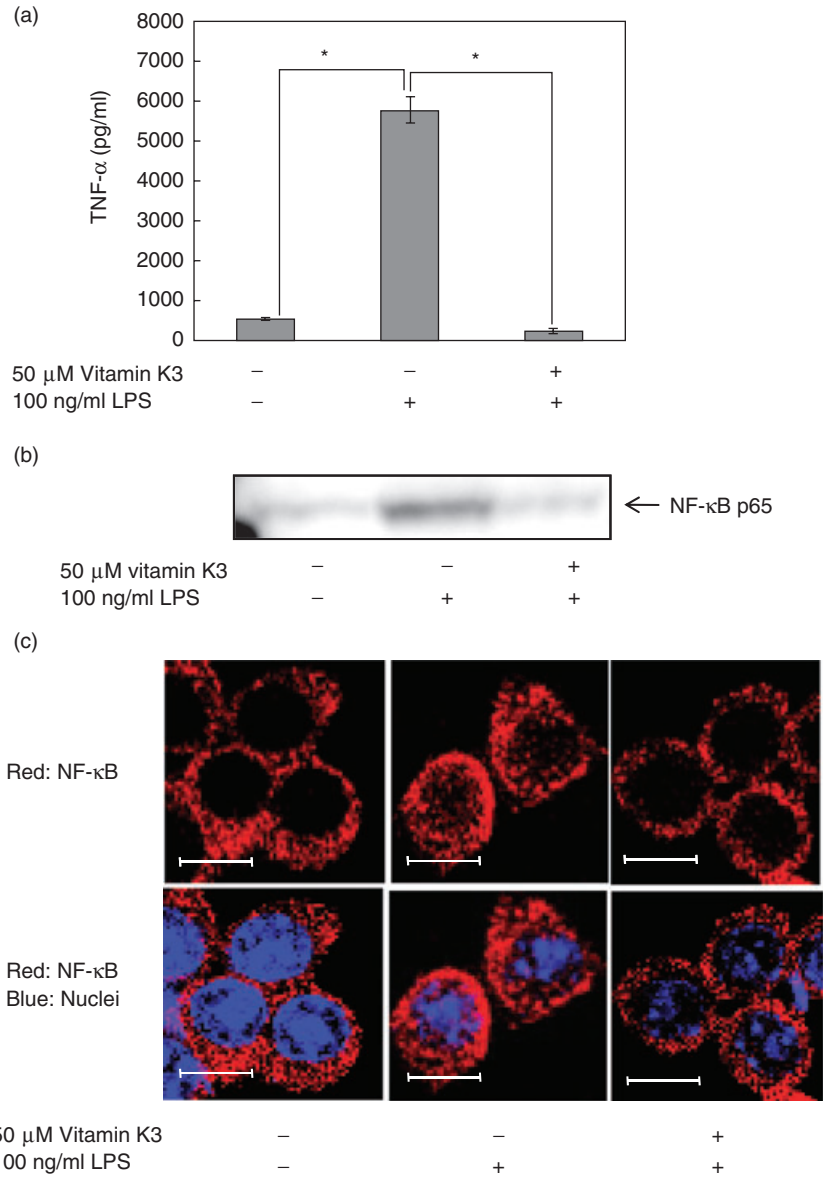

Fig. 3. Inhibitory effects of vitamin K_3 on the lipopolysaccharide (LPS)-induced tumour necrosis factor (TNF)-α production and nuclear factor (NF)-κB nuclear translocation in RAW264·7 cells. (a) RAW264·7 cells were pretreated with 50 μM vitamin K_3 or DMSO as a vehicle control for 30 min and then treated with 100 ng/ml LPS for 24 h, and the TNF-α concentration in the cell medium was measured by enzyme-linked immunosorbent assay. Each data value is expressed as mean ± standard error of duplicates of three experiments. The presence of significant differences is indicated by asterisks ($P < 0{\cdot}05$). (b) RAW264·7 cells were incubated with 50 μM vitamin K_3 or dimethylsulphoxide (DMSO) as a vehicle control for 30 min, followed by treatment with 100 ng/ml LPS for 30 min. The nuclear proteins were prepared from the cells and were subjected to Western blot analysis for evaluation of the nuclear translocation of NF-κB p65. Typical pictures are shown from at least triplicate determinations. (c) RAW264·7 cells were treated as described in Fig. 3b, and the nuclear translocation of NF-κB p65 was assessed by immunofluorescence microscopy (red; NF-κB; blue: nuclei). Typical pictures are shown from at least triplicate determinations. Scale bar, 10 μm.

inflammatory effects [11,12]. Therefore, the inhibitory effect of vitamins K_1 and K_2 on the NF-κB p65 nuclear translocation was compared with that of vitamin K_3 (Fig. 2d). Under our experimental conditions, vitamins K_1 and K_2 were not able to inhibit the nuclear translocation of NF-κB p65, unlike vitamin K_3.

Inhibitory effects of vitamin K_3 on the LPS-evoked NF-κB p65 nuclear translocation and TNF-α production in RAW 264·7 cells

LPS, a membrane constituent of Gram-negative bacteria and a bacterial endotoxin, is a potent stimulator of inflammatory responses. LPS also induces the NF-κB activation via Toll-like receptor 4 and causes the following production of various cytokines [13]. Therefore, we investigated whether vitamin K_3 was able to suppress the LPS-induced production of TNF-α in RAW264·7 cells (Fig. 3a). LPS at the concentration of 100 ng/ml produced 5769 pg/ml TNF-α, and 50 μM vitamin K_3 decreased the LPS-evoked TNF-α production to 232 pg/ml. Next, the inhibitory effects of vitamin K_3 on the LPS-caused NF-κB p65 nuclear translocation were evaluated in RAW264·7 cells. In Western blot analysis (Fig. 3b) and the immunofluorescence study (Fig. 3c), vitamin K_3 suppressed completely the LPS-induced nuclear translocation of NF-κB p65.

Inhibitory effects of vitamin K_3 on the LPS-evoked NF-κB nuclear translocation and TNF-α production in peritoneal macrophages

To confirm the anti-inflammatory effects of vitamin K_3 *in vivo*, peritoneal macrophages were prepared from C57BL/6

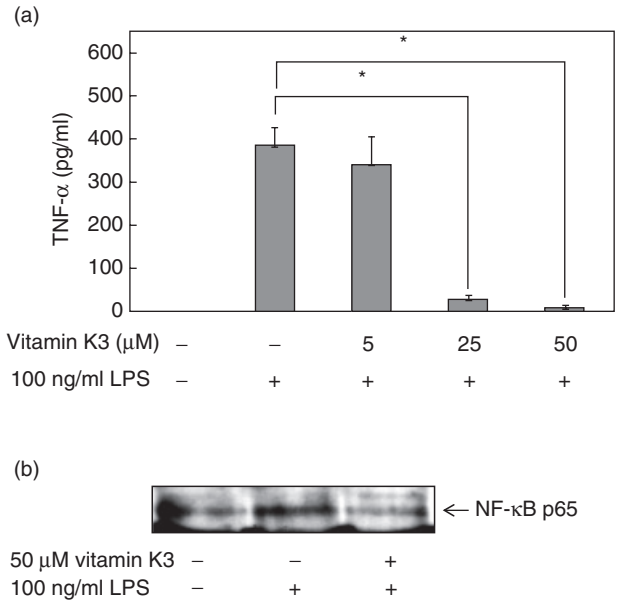

Fig. 4. Inhibitory effects of vitamin K_3 on the lipopolysaccharide (LPS)-induced tumour necrosis factor (TNF)-α production and nuclear factor (NF)-κB p65 nuclear translocation in peritoneal macrophages. (a) Peritoneal macrophages were prepared from C57BL/6 mice and were incubated with 5, 10 or 50 μM vitamin K_3 or dimethylsulphoxide (DMSO) as a vehicle control for 30 min. The cells were then treated with 100 ng/ml LPS for 24 h, and the TNF-α concentration in the cell medium was measured by enzyme-linked immunosorbent assay. Each data value is expressed as mean ± standard error of duplicates of three experiments. The presence of significant differences is indicated by asterisks ($P < 0{\cdot}05$). (b) Peritoneal macrophages were incubated with 50 μM vitamin K_3 or DMSO as a vehicle control for 30 min, followed by treatment with 100 ng/ml LPS for 30 min. The nuclear proteins were prepared from the cells and were subjected to Western blot analysis for evaluation of the nuclear translocation of NF-κB p65. Typical pictures are shown from at least triplicate determinations.

mice, and the dose-dependent suppressive effect of vitamin K_3 on the TNF-α production was evaluated in peritoneal macrophages (Fig. 4a). As a result, 25 and 50 μM vitamin K_3 inhibited significantly the 100 ng/ml LPS-induced TNF-α production, although 5 μM vitamin K_3 had no effect. In Western blot analysis, it was also confirmed that vitamin K_3 could suppress the LPS-induced nuclear translocation of NF-κB p65 in peritoneal macrophages (Fig. 4b).

Anti-inflammatory effects of vitamin K_3 in the ARDS model

Administration of high dose LPS causes ARDS, which occurs in the setting of acute severe illness complicated by systemic inflammation [6]. Therefore the mice were injected intraperitoneally with 15 mg/kg BW LPS, and the mouse model of ARDS was developed. To evaluate the suppressive effects of vitamin K_3 in the LPS-induced ARDS mouse model, the mice were injected intraperitoneally with 15 mg/kg BW LPS 30 min after 100 mg/kg BW vitamin K_3 administration. Administration of 15 mg/kg BW LPS led to a significant increase in the serum TNF-α concentration to 2828 pg/ml, and vitamin K_3 reduced significantly the serum TNF-α level (Fig. 5a). Next, we investigated whether vitamin K_3 was able to attenuate the severity of lung injury in the ARDS model (Fig. 5b). In mice treated with 15 mg/kg BW LPS, lung injury including intra-alveolar haemorrhaging, interstitial oedema, alveolar collapse and massive inflammatory cells infiltration was confirmed by H&E staining using light microscopy. In the group that was treated with 100 mg/kg BW vitamin K_3 and 15 mg/kg BW LPS, the disruption of lung tissue was attenuated in comparison with that of LPS alone. The suppressive effects of vitamin K_3 in the ARDS model were also examined quantitatively. The total cell count in the BAL fluid was increased approximately 25-fold 24 h after administration of 15 mg/kg BW LPS, and the number of neutrophils in BAL was also increased significantly. Treatment of 100 mg/kg BW vitamin K_3 reduced significantly the LPS-evoked increases in the total cell and neutrophil counts in the BAL fluid (Fig. 5c,d). To elucidate the mechanism underlying the attenuation of LPS-evoked lung injury by vitamin K_3, the inhibitory effect of vitamin K_3 on the nuclear translocation of NF-κB p65 in lung tissue was examined by Western blot analysis. As shown in Fig. 5e, vitamin K_3 suppressed the LPS-induced nuclear translocation of NF-κB p65.

Because it was found that vitamin K_3 could exert the suppressive effects in the ARDS model (Fig. 5), the therapeutic effects of vitamin K_3 in the ARDS model are also expected. To evaluate the therapeutic effects of vitamin K_3 in the LPS-induced ARDS mouse model, the mice were injected intraperitoneally with 100 mg/kg BW vitamin K_3 30 min after 15 mg/kg BW LPS administration. First, we investigated whether the post-treatment of vitamin K_3 was able to attenuate the severity of lung injury in the LPS-induced ARDS model. In the group treated with 15 mg/kg BW LPS and 100 mg/kg BW vitamin K_3, the disruption of lung tissue was attenuated in comparison with the group of LPS alone (Fig. 6a). In addition, 100 mg/kg BW vitamin K_3 could reduce significantly the LPS-caused increases in both the total cell and neutrophil counts in the BAL fluid (Fig. 6b,c).

Discussion

Vitamin K, an essential nutrient, has an important role in normal blood coagulation systems via post-translational modification, and acts as a co-factor of various plasma proteins such as prothrombin, factor VII, factor IX and factor X [14]. Recently, various chemopreventive activities of vitamin K were also reported. In a rat paw oedema model, vitamin K_1 and the seven carbon aliphatic carboxylic acid catabolic products from vitamin K were more potent than either phenylbutazone or Saridone® (a mixed analgesic preparation) in ameliorating inflammation and pain

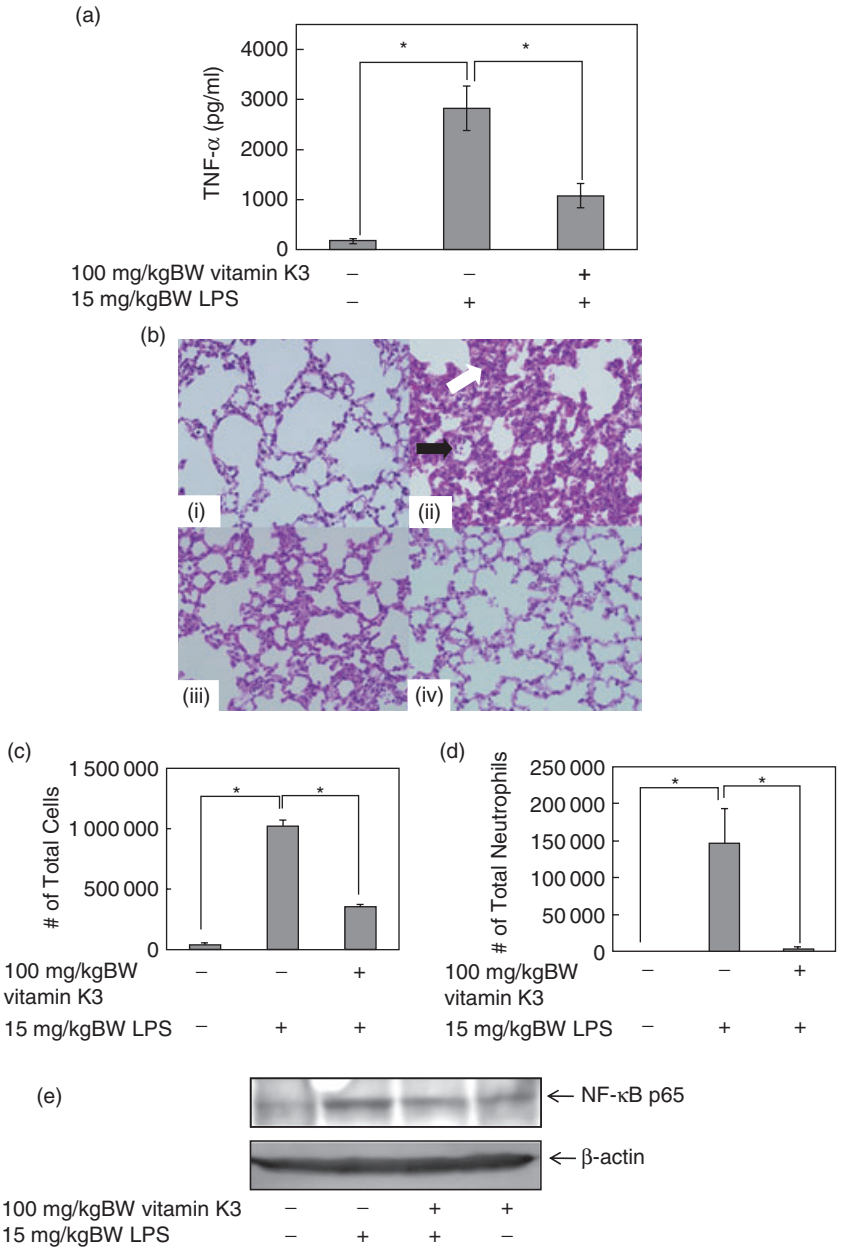

Fig. 5. Suppressive effects of vitamin K_3 in the lipopolysaccharide (LPS)-induced acute respiratory distress syndrome (ARDS) mouse model. Male C57BL/6 mice were injected intraperitoneally with 100 mg/kg body weight (BW) vitamin K_3 or corn oil as vehicle control. After 30 min, the mice were injected intraperitoneally with 15 mg/kg BW LPS or saline as a vehicle control. (a) After 1 h, the mice were killed, and the serum tumour necrosis factor (TNF)-α concentration was measured by enzyme-linked immunosorbent assay. Each data value is expressed as mean ± standard error (s.e.), $n = 3$. The presence of significant differences is indicated by asterisks ($P < 0{\cdot}05$). (b) After 6 h, the mice were killed, and the lung tissue was collected. The lung tissue was stained with haematoxylin and eosin (H&E ×400), and histopathological examination of the lung tissue was performed. Typical pictures are shown from at least triplicate determinations. (i) Negative control group; (ii) LPS group; (iii) vitamin K_3+LPS group; (iv) vitamin K_3 group. The white arrow indicates oedematous change in the alveolar wall and the swelling of alveolar epithelial cells, and the black arrow indicates massive polymorphonuclear infiltration. (c,d) After 24 h the mice were killed, and total cell (c) and neutrophil (d) counts in the broncoalveolar lavage (BAL) fluid were performed. Each data value is expressed as mean ± s.e., $n = 3$. The presence of significant differences is indicated by asterisks ($P < 0{\cdot}05$). (e) After 6 h the mice were killed and the lung tissue was collected. Nuclear proteins were prepared from lung tissue and were subjected to Western blot analysis to evaluate the nuclear translocation of NF-κB p65, and the β-actin protein was detected as the internal standard. Typical pictures are shown from at least triplicate determinations.

[15,16]. Menaquinone-4, a vitamin K_2 congener, was reported to be capable of modulating osteoblast proliferation [17], alkaline phosphatase activity [17] and cyclooxygenase activity [18]. In a chronic antigen-induced rabbit model of arthritis, the oral administration of vitamin K_3 was effective in inhibiting synovitis [19]. Thus, vitamin K is a possible candidate as a chemopreventive agent against various diseases.

In this study, we found that vitamin K_3 was able to attenuate the lung injury and inhibit NF-κB activation in the ARDS mouse model (Figs 5 and 6). The pathogenesis of ARDS is complex and involves multiple signal transduction processes. Particular attention has been given to the NF-κB signalling, which is required for expression of various cytokines and the following inflammatory responses [20]. The degree of NF-κB activation was reported to increase in patients with sepsis or acute lung injury [21–24], and the nuclear accumulation of NF-κB p65 was observed in alveolar macrophages from patients with acute lung injury caused by severe infection, in contrast to alveolar macrophages from control patients [21,22]. The increased nuclear levels of NF-κB p65 were also confirmed in peripheral blood mononuclear cells (PBMCs) from patients with sepsis [23,24]. In addition, it appears that acute lung injury patients who do not survive exert greater and more prolonged NF-κB activation than the surviving patients [23,24]. These results suggest that NF-κB activation is central to the development of pulmonary inflammation

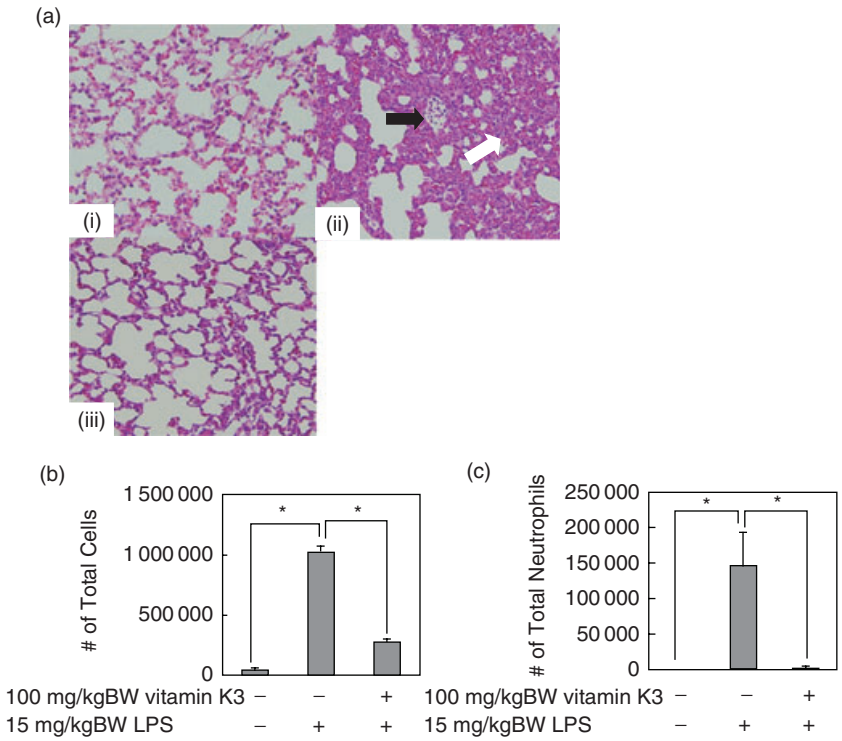

Fig. 6. Therapeutic effects of vitamin K_3 in the lipopolysaccharide (LPS)-induced acute respiratory distress syndrome (ARDS) mouse model. Male C57BL/6 mice were injected intraperitoneally with 15 mg/kg body weight (BW) LPS or saline as a vehicle control. After 30 min, the mice were injected intraperitoneally with 100 mg/kg BW vitamin K_3 or corn oil as vehicle control. (a) After 6 h the mice were killed, and the lung tissue was collected. The lung tissue was stained with haematoxylin & eosin (H&E ×400), and histopathological examination of the lung tissue was performed. Typical pictures are shown from at least triplicate determinations. (i) Negative control group; (ii) LPS group; (iii) LPS+vitamin K_3 group. The white arrow indicates oedematous change in the alveolar wall and the swelling of alveolar epithelial cells, and the black arrow indicates massive polymorphonuclear infiltration. (b,c). After 24 h the mice were killed, and total cell (b) and neutrophil (c) counts in the bronchoalveolar lavage (BAL) fluid were performed. Each data value is expressed as mean ± standard error, $n = 3$. The presence of significant differences is indicated by asterisks ($P < 0{\cdot}05$).

and acute lung injury. In cell culture experiments using HEK293 cells and RAW264·7 cells, vitamin K_3 was able to inhibit the NF-κB p65 activation induced by both TNF-α stimulation (Fig. 2a,b) and LPS stimulation (Fig. 3b,c). Vitamin K_3 was also able to reduce the DNA binding activity of NF-κBs in HEK293 cells (Fig. 2c). TNF-α activates the NF-κB signalling pathway via the binding of TNF-α to TNFR [10]. On the other hand, LPS-evoked NF-κB activation begins with the binding of LPS to Toll-like receptor 4 [13]. Thus, the NF-κB signalling pathway has multiple first steps. Therefore, the pathway common to both TNF-α and LPS-dependent NF-κB signalling pathways may be the action site of vitamin K_3. A recent study revealed that vitamin K_2 inhibited cytokine-induced NF-κB activation through suppression of IκB kinase (IKK) activity [25]. IKK is associated with both TNF-α and LPS-dependent NF-κB signalling pathways, and vitamin K_3 may also be able to inhibit NF-κB activation via suppressing the IKK activity. Anti-inflammatory activity was also reported to be linked to anti-oxidative activity [26], and the reactive oxygen species (ROS) and redox pathways can modulate the NF-κB signalling [27]. Vitamin K_3 is the effective regulator for ROS generation and the redox system. In fact, vitamin K_3 inhibited the tumour cell growth through oxidative stress via redox cycling of quinine to produce ROS [28,29]. Therefore, there is a possibility that vitamin K_3 exerted the inhibitory effects on the NF-κB signalling through acting to the ROS and/or redox pathways.

Under our experimental conditions, vitamin K_3 inhibited NF-κB activation more potently than vitamins K_1 or K_2 (Fig. 2d), although vitamins K_1 and K_2 were reported to have anti-inflammatory activity [11] and the suppressive effects of vitamin K_2 against the NF-κB activity were observed [26]. These results mean that our data were different from the previous reports. In a previous study, the 24-h treatment of vitamin K_2 led to suppression of NF-κB activation in hepatoma cells [24]. In our experiments, vitamin K_2 could not inhibit the NF-κB p65 nuclear translocation 1 h after the treatment in HEK293 cells (Fig. 2d). These results suggest that vitamin K_2 itself may have slight anti-inflammatory effects and its metabolized form may be a strong anti-inflammatory agent.

In conclusion, our data suggest that vitamin K_3 can attenuate NF-κB-dependent inflammatory responses and related inflammatory diseases, such as ARDS. Little therapeutic progress has been made, although it was reported that some compounds, such as liquorice flavonoids, SB203580 p38MAPK specific inhibitor and Gardenia-awetu compounds, were able to attenuate acute lung injury/ARDS [30–32], and the mortality rate of ARDS remains high. Therefore, usage of vitamin K_3 may be an effective therapeutic strategy against acute lung injury/ARDS.

Acknowledgements

This work was supported, in part, by a grant for the Global COE Program 'Global Center of Excellence for Education and Research on Signal Transduction Medicine in the Coming Generation' from the Ministry of Education, Culture, Sports, Science and Technology of Japan (M. Y.). This work was also supported by a grant for the Education Program for Specialized Clinician in the Support Program for Improving Graduate School Education from the Ministry of Education, Culture, Sports, Science and Technology of Japan (S. T.).

Disclosure

All authors declare there is no conflict of interest.

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