

Original Article

Vitamin K₂ suppresses rotenone-induced microglial activation *in vitro*

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Aim: Increasing evidence has shown that environmental factors such as rotenone and paraquat induce neuroinflammation, which contributes to the pathogenesis of Parkinson's disease (PD). In this study, we investigated the molecular mechanisms underlying the repression by menaquinone-4 (MK-4), a subtype of vitamin K₂, of rotenone-induced microglial activation *in vitro*.

Methods: A microglial cell line (BV2) was exposed to rotenone (1 μmol/L) with or without MK-4 treatment. The levels of TNF-α or IL-1β in 100 μL of cultured media of BV2 cells were measured using ELISA kits. BV2 cells treated with rotenone with or without MK4 were subjected to mitochondrial membrane potential, ROS production, immunofluorescence or immunoblot assays. The neuroblastoma SH-SY5Y cells were treated with conditioned media (CM) of BV2 cells that were exposed to rotenone with or without MK-4 treatment, and the cell viability was assessed using MTT assay.

Results: In rotenone-treated BV2 cells, MK-4 (0.5–20 μmol/L) dose-dependently suppressed the upregulation in the expression of iNOS and COX-2 in the cells, as well as the production of TNF-α and IL-1β in the cultured media. MK-4 (5–20 μmol/L) significantly inhibited rotenone-induced nuclear translocation of NF-κB in BV2 cells. MK-4 (5–20 μmol/L) significantly inhibited rotenone-induced p38 activation, ROS production, and caspase-1 activation in BV2 cells. MK-4 (5–20 μmol/L) also restored the mitochondrial membrane potential that had been damaged by rotenone. Exposure to CM from rotenone-treated BV2 cells markedly decreased the viability of SH-SY5Y cells. However, this rotenone-activated microglia-mediated death of SH-SY5Y cells was significantly attenuated when the BV2 cells were co-treated with MK-4 (5–20 μmol/L).

Conclusion: Vitamin K₂ can directly suppress rotenone-induced activation of microglial BV2 cells *in vitro* by repressing ROS production and p38 activation.

Keywords: Parkinson's disease; rotenone; vitamin K₂; microglia; BV2 cells; SH-SY5Y cells; cytokines; ROS; mitochondrial membrane potential; p38; neuroinflammation

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Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disorder and is characterized by the preferential loss of dopaminergic (DA) neurons in the substantia nigra pars compacta (SNpc). Both genetic and environmental factors can cause PD^[1–3]. Multiple cellular alterations are associated with PD pathogenesis, including the accumulation of toxic proteins^[4], oxidative stress^[5], mitochondrial dysfunction^[6] and neuroinflammation^[7]. It is well documented that

environmental factors such as pesticides are causal factors for the development of PD^[8,9]. Epidemiological studies also suggest that exposure to some toxic agents can induce microglial activation and mitochondrial dysfunction, which are currently recognized as prominent features of PD^[10,11].

Rotenone, a widely used pesticide, can selectively inhibit complex I of the mitochondrial electron transport chain, thereby increasing the production of free hydroxyl radicals and inducing oxidative stress^[12]. Rotenone freely crosses cellular membranes and accumulates throughout the brain to impair the mitochondrial function of neurons, particularly DA neurons, leading to neurodegeneration^[13,14]. Thus, rotenone-treated animals effectively reproduce the pathological features of PD with the loss of SNpc DA neurons^[15]. Our

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previous study demonstrates that rotenone can act directly on microglial mitochondria to activate microglia and to induce the expression of inflammatory factors via the nuclear factor kappa B (NF- κ B) signaling pathway^[16]. Thus, rotenone not only damages DA neurons but also activates microglia by inhibiting mitochondrial complex I.

Interestingly, in the SNpc of PD patients, DA neuronal degeneration is always accompanied by the presence of activated microglia^[7, 17]. In a PD animal model induced by rotenone, activated microglia are also observed^[18]. Microglia are a type of glial cell that act as the first and main form of active immune defense in the central nervous system^[19] and play an important role in neuroinflammation, a process that is associated with the pathogenesis of PD^[20]. Both postmortem brains and various animal models of PD show massive amounts of activated microglia around degenerated neurons^[21]. The activated microglia produce a wide array of prostanoids, free radicals and cytokines, leading to neurodegeneration^[22]. Individuals who take non-steroidal anti-inflammatory drugs show a reduced risk of developing PD^[23]. Therefore, neuroinflammation is closely associated with PD development.

Vitamin K is recognized as a cofactor in the synthesis of the Gla-protein family and is also known to play key roles in physiological regulation, such as cardiovascular and bone metabolism functions^[24, 25]. Vitamin K compounds include two naturally occurring forms: vitamin K₁ (phylloquinone), which is mainly found in green vegetables, such as spinach, broccoli, kale and Brussels sprouts, and vitamin K₂ (menaquinone or MK-n), which is produced mainly by microorganisms. In the human intestine, bacteria convert vitamin K₁ into vitamin K₂ to meet our daily needs. Menaquinone-4 (MK-4), a subtype of vitamin K₂ that contains a geranylgeranyl group (isoprenyl side chain) at the 3-position of 2-methyl-1,4-naphthoquinone, is derived from the diet, either from animal origin or synthesized from other vitamin K analogues^[26, 27]. Increasing evidence suggests that vitamin K₂ is associated with inflammatory regulation^[28, 29]. However, the mechanisms by which vitamin K₂ influences neuroinflammation are still unclear. Recent studies indicate that vitamin K₂ serves as a mitochondrial electron carrier that can rescue mitochondrial dysfunction^[30], suggesting that vitamin K₂ has the potential for protecting mitochondria.

Here, we demonstrate an inhibitory effect of MK-4 on rotenone-induced microglial activation by restoration of the mitochondrial membrane potential, thereby decreasing reactive oxygen species (ROS) production and inhibiting NF- κ B activation. In addition, MK-4 represses microglial activation-mediated neuronal cell death.

Materials and methods

Reagents

Rotenone (Sigma-Aldrich, Saint Louis, MO, USA) and MK-4 (Sigma-Aldrich) were dissolved in DMSO and 99.5% ethanol as stock solutions, respectively. Before use, the stock (50 mmol/L) was diluted with culture media, in which the final DMSO concentration was lower than 0.01%, and the ethanol

concentration was lower than 0.2%.

Cell culture and treatment

BV2 cells, a mouse microglial cell line (a kind gift from Dr Jian-qing DING at Shanghai Jiao Tong University, China)^[31], were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA) with 100 μ g/mL penicillin and 100 μ g/mL streptomycin (Invitrogen, Carlsbad, CA, USA). BV2 cells were treated with rotenone at a concentration of 1 μ mol/L with MK-4 at concentrations of 0.5, 1, 5, 10, or 20 μ mol/L for 24 h. The cells were washed with culture media and then cultured for another 24 h to produce the conditioned media (CM).

Measurement of ROS generation

A reactive oxygen species assay kit (Beyotime, Shanghai, China) was used to detect the intracellular generation of ROS. The cells were incubated in serum-free media with 10 μ mol/L 2',7'-dichlorofluorescein diacetate (DCF-DA) (Invitrogen) at 37°C for 20 min. Next, the cells were washed three times with serum-free medium. Samples were analyzed at an excitation wavelength of 488 nm and an emission wavelength of 522 nm using a flow cytometer (Beckman Coulter, Kraemer Boulevard Brea, CA, USA).

Measurement of caspase-1 activity

The Green FLICA™ caspase-1 assay kit (ImmunoChemistry Technologies, Bloomington, MN, USA) was used to detect caspase-1 activation. Cells were incubated with ICT'S green caspase-1 inhibitor probe, FAM-YVAD-FMK, for 1 h at 37°C. The cells were observed under an inverted IX71 microscope system (Olympus, Tokyo, Japan), and the fluorescence intensity of caspase-1 was analyzed using a multi-detection reader (Molecular Devices) at an excitation of 492 nm and emission of 520 nm.

Measurement of the mitochondrial membrane potential

For the measurement of the mitochondrial membrane potential ($\Delta\Psi$ m), tetramethylrhodamine methyl ester (TMRM) (Sigma-Aldrich) was added to the cell culture medium for 15 min^[32]. To identify the presence of viable mitochondria, the cells were double stained with MitoTracker Green (Beyotime). The cells were then observed under an inverted IX71 microscope system (Olympus). The fluorescence intensity of TMRM was analyzed using a multi-detection reader (Molecular Devices, Sunnyvale, CA, USA) at an excitation of 549 nm and emission of 575 nm.

Subcellular fractionation assay for nuclear extraction

BV2 cells were lysed in a fractionation buffer containing 320 mmol/L sucrose, 3 mmol/L CaCl₂, 2 mmol/L MgAc, 0.1 mmol/L EDTA, 1 mmol/L DTT, 0.5 mmol/L PMSF and 0.5% NP-40 for 20 min on ice. The cells were centrifuged at 600 \times g for 15 min at 4°C, and then the supernatant was collected as the cytoplasmic fraction. The pellet was washed once with the

fractionation buffer without NP-40, and then lysed in a nuclear lysis buffer containing 20 mmol/L HEPES (pH 7.9), 25% glycerol, 1.5 mmol/L MgCl₂, 280 mmol/L KCl, 0.2 mmol/L EDTA, 1 mmol/L DTT, 0.5 mmol/L PMSF, and 0.3% NP-40.

Immunoblot analysis and antibodies

The BV2 cells were lysed in a 1×SDS lysis buffer (25 mmol/L Tris-HCl, pH 7.6, 150 mmol/L NaCl, 1% NP-40, and 1% sodium deoxycholate) in the presence of a protease inhibitor cocktail (Roche, Mannheim, Germany). Proteins were separated by 12% SDS-PAGE and then transferred onto a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA). An immunoblot analysis was performed using the following primary antibodies: monoclonal anti-GAPDH antibody was from Millipore; monoclonal anti-p38 antibody was from Santa Cruz Biotechnology (Dallas, Texas, USA); polyclonal anti-iNOS antibodies were from ABCAM (Cambridge, UK); polyclonal anti-COX2, anti-histone 2B, anti-IκB, and anti-p65 antibodies were from Epitomics (Cambridge, UK); polyclonal anti-IKK, anti-p-IKK (176/180), and anti-p-p38 antibodies were from Cell Signaling Technology (Danvers, MA, USA). The secondary antibodies, sheep anti-mouse or anti-rabbit IgG-HRP, were purchased from GE Healthcare (Beijing, China).

ELISA assay for TNF-α and IL-1β

The levels of TNF-α or IL-1β in 100 μL of cultured media from BV2 cells were measured using ELISA kits (R&D Systems, Shanghai, China) according to the manufacturer's instructions.

Cell viability assay

Cell viability was assessed using the MTT assay. The BV2 cells were seeded and treated with different concentrations of MK-4 for 24 h. For conditioned medium assays, SH-SY5Y cells were treated with CM from rotenone- and MK-4-treated BV2 cells for 24 h. The cells were incubated with 0.5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) at 37°C for 2 h. Then, 150 μL of dimethylsulfoxide was added to stop the reaction. The absorbance was measured at 570 nm to determine cell viability.

Propidium iodide staining

SH-SY5Y cells treated with CM were incubated with propidium iodide (PI, Beyotime) for 20 min away from light and visualized using an inverted IX71 microscope system (Olympus).

Immunocytofluorescence

The treated BV2 cells were washed with PBS and fixed with 4% paraformaldehyde in PBS (pH 7.4) for 5 min. Then, the cells were treated with 0.25% Triton X-100 for 5 min and blocked with 4% FBS in PBST. Immunocytochemical staining was performed using polyclonal anti-p65 antibodies. The cells were then incubated with Alexa Fluor 594 donkey anti-rabbit secondary antibodies (Jackson Immuno Research, PA, USA) and DAPI (Invitrogen, Carlsbad, CA, USA).

Statistical analysis

Statistical comparisons between groups and treatments were performed using a one-way analysis of variance (ANOVA). Student's *t*-tests were used for comparing two groups. A *P* value of <0.05 was considered statistically significant. The data are presented as the mean±SEM.

Results

MK-4 suppressed the rotenone-induced production of inflammatory factors in BV2 cells

Because our previous studies showed that rotenone induces the production of inflammatory factors in BV2 cells^[16], and it has been reported that MK-4 represses the production of inflammatory factors^[33], we tested whether MK-4 could inhibit the rotenone-induced production of inflammatory factors in BV2 cells. We first examined whether MK-4 affects BV2 cell viability. MK-4 itself did not influence BV2 cell viability at concentrations up to 100 μmol/L (Figure 1A). Because MK-4 represses LPS-induced inflammatory factor production at concentrations of 1, 10 or 25 μmol/L^[33, 34], we decided to perform experiments using concentrations of MK-4 that ranged from 0 μmol/L to 20 μmol/L in order to examine its effects on rotenone-induced microglial activation. In BV2 cells that were treated with rotenone, the inflammatory factors iNOS and COX-2 were significantly increased (Figure 1B). The production of IL-1β (Figure 1C) and TNF-α (Figure 1D) was also significantly increased. However, the rotenone-induced increase of inflammatory factors was significantly blocked by MK-4 in a dose-dependent manner (Figure 1B, 1C, and 1D), suggesting that MK-4 can inhibit the production of inflammatory factors induced by rotenone in BV2 cells.

MK-4 inhibited the rotenone-induced nuclear translocation of the NF-κB p65 subunit in BV2 cells

NF-κB is an important transcription factor that plays a key role in transactivating inflammatory gene expression by its translocation to the nucleus when microglia are exposed to stimulation^[16, 35, 36]. Because rotenone activates NF-κB in microglial cells^[16], and MK-4 inhibits the production of NF-κB-targeted inflammatory factors (Figure 1), we wondered whether MK-4 could inhibit NF-κB nuclear translocation. We first examined the location of the NF-κB p65 subunit. p65 was located in the cytoplasm under normal conditions, whereas it was partially translocated to the nucleus after 1 μmol/L rotenone treatment in BV2 cells (Figure 2A). However, p65 was predominantly located in the cytoplasm after MK-4 treatment at doses greater than 1 μmol/L (Figure 2A), suggesting that MK-4 inhibits rotenone-induced nuclear translocation of NF-κB. To further verify the inhibition of NF-κB nuclear translocation by MK-4, we performed a fractionation assay. Consistent with immunocytochemical data, the nuclear level of p65 was significantly decreased after MK-4 treatment in rotenone-treated BV2 cells (Figure 2B). Thus, our data suggest that MK-4 inhibits rotenone-induced NF-κB nuclear translocation in BV2 cells.

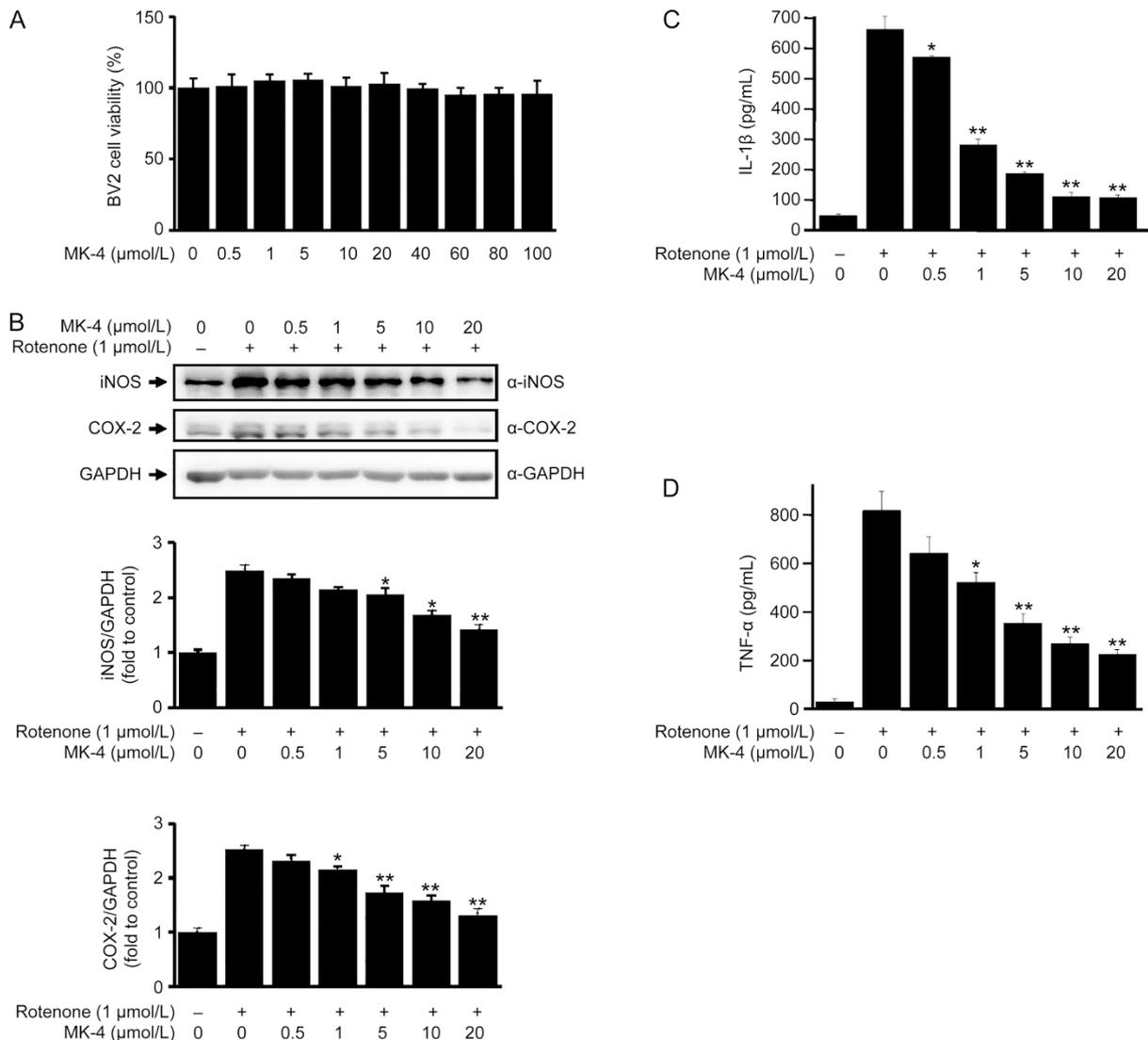


Figure 1. MK-4 suppresses rotenone-induced inflammatory factor expression. (A) Various doses (0.5–100 μmol/L) of MK-4 were administered to BV2 cells for 24 h. Cell viability was measured by the MTT assay. No significant changes were observed in any group. (B) BV2 cells were treated with different doses of MK-4 and 1 μmol/L rotenone for 24 h. An immunoblot analysis showed the expression of iNOS and COX-2, with antibodies used as indicated. The band intensities of iNOS and COX-2 relative to that of GAPDH are shown in the lower two panels. The value of the group without drug (dissolvent only) is normalized as 1. The data are presented as the mean±SEM from three independent experiments. **P*<0.05, ***P*<0.01 vs the group in which the cells were treated with 1 μmol/L rotenone and 0 μmol/L MK-4, as analyzed by one-way ANOVA. (C and D) The levels of IL-1β (C) and TNF-α (D) in the cultured media were measured using ELISA assays. The data are presented as the mean±SEM from three independent experiments. **P*<0.05, ***P*<0.01 vs the group in which the cells were treated with 1 μmol/L rotenone and 0 μmol/L MK-4, as analyzed by one-way ANOVA.

MK-4 suppressed the rotenone-induced degradation of IκB by inhibiting IKK and p38 activation

IκB, an inhibitor of NF-κB, is ubiquitinated and subsequently rapidly degraded by the ubiquitin-proteasomal system after its phosphorylation by IκB kinase (IKK), leading to the activation of NF-κB^[37, 38]. Because we have shown that MK-4 inhibits rotenone-induced nuclear translocation of the NF-κB subunit p65, we wondered whether MK-4 affects IκB stability. In BV2 cells that were treated with rotenone, IκB was obviously degraded (Figure 3A), and phospho-IKK (p-IKK) was significantly increased (Figure 3B). However, the degradation of IκB

and the activation of IKK were significantly inhibited by MK-4 treatment (Figure 3A and 3B).

Previous studies have shown that LPS- or rotenone-induced NF-κB activation is dependent on the phosphorylation of p38^[16, 39, 40]. We therefore examined whether MK-4 affects p38 phosphorylation. In BV2 cells that were treated with rotenone, the phosphorylation of p38 was increased after rotenone treatment; however, the phosphorylation of p38 was significantly decreased by MK-4 in a dose-dependent manner (Figure 3C).

Many studies have reported that p38 is phosphorylated in response to reactive oxygen species (ROS) and that rotenone

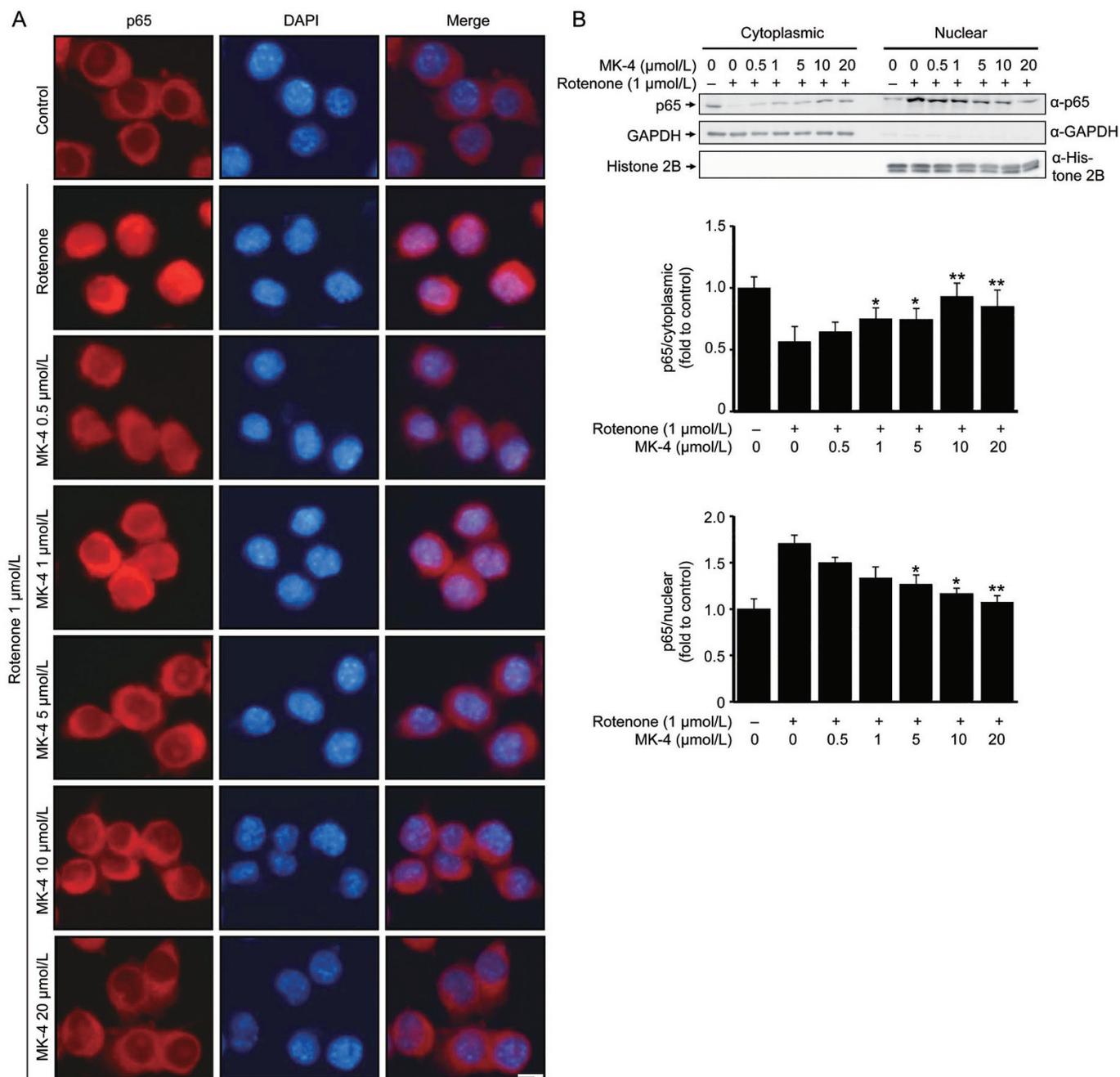


Figure 2. MK-4 inhibits rotenone-induced nuclear accumulation of the NF- κ B p65 subunit in BV2 cells. (A) Various doses of MK-4 and 1 μ mol/L rotenone were administered to BV2 cells as indicated for 6 h. The cells were fixed and labeled with anti-p65 (red) antibodies. Then, the cells were visualized under a fluorescent microscope. The nuclei were stained with DAPI (1 μ g/mL) (blue). Scale bars, 5 μ m. (B) The cytoplasmic and nuclear fractions from the treated BV2 cells were immunoblotted with anti-p65, anti-GAPDH or anti-histone 2B antibodies. The band intensities of p65 relative to that of GAPDH (cytoplasmic fraction) or histone 2B (nuclear fraction) are shown in the lower two panels. The value of the group without drug (dissolvent only) is normalized as 1. The data are presented as the mean \pm SEM from three independent experiments. * P <0.05, ** P <0.01 vs the group in which the cells were treated with 1 μ mol/L rotenone and 0 μ mol/L MK-4, as analyzed by one-way ANOVA.

can induce mitochondrial ROS production^[41,42]. We wondered whether the decreased p38 activation induced by MK-4 in rotenone-treated BV2 cells is associated with ROS production. After the rotenone and MK-4 treatment, cells were incubated with DCF-DA, an ROS indicator that is oxidated by ROS

into DCF, a highly fluorescent compound. In BV2 cells, rotenone significantly induced ROS production. However, ROS production was obviously attenuated after MK-4 treatment (Figure 3D).

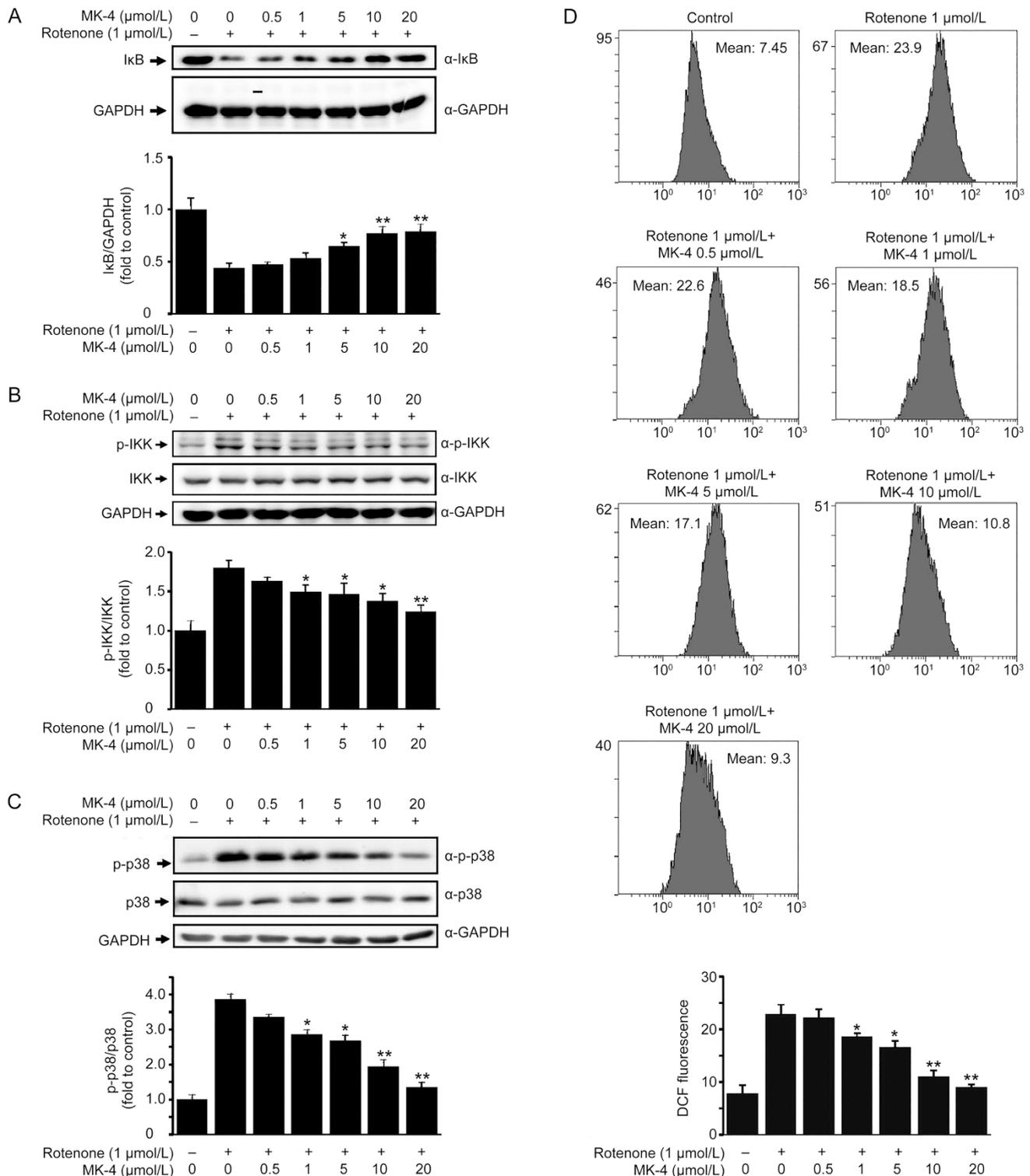


Figure 3. MK-4 suppresses rotenone-induced degradation of IκB, activation of IKK and p38, and production of ROS. (A) BV2 cells were treated with different doses of MK-4 and 1 μmol/L rotenone for 24 h as indicated. The cells lysates were immunoblotted with anti-IκB antibodies. GAPDH served as the loading control. The band intensities of IκB relative to that of GAPDH are shown in the lower panel. The data are presented as the mean±SEM from three independent experiments. **P*<0.05, ***P*<0.01 vs the group in which the cells were treated with 1 μmol/L rotenone and 0 μmol/L MK-4, analyzed by one-way ANOVA. (B) The cells lysates were immunoblotted with anti-p-IKK or anti-IKK antibodies. A quantitative analysis was performed as described in (A). (C) The cell lysates were immunoblotted with anti-p-p38 or anti-p38 antibodies. A quantitative analysis was performed as described in (A). (D) The cells were stained with an ROS indicator, DCF-DA, for 20 min. The DCF fluorescence of the cells was evaluated using a flow cytometer. The data are presented as the mean±SEM from three independent experiments. **P*<0.05, ***P*<0.01 vs the group in which the cells were treated with 1 μmol/L rotenone and 0 μmol/L MK-4, as analyzed by one-way ANOVA.

MK-4 decreased rotenone-induced caspase-1 activation in BV2 cells

Previous studies have reported that the activated NF- κ B signaling pathway may induce inflammasome activation by elevating the expression of inflammasome components^[43]. The assembled inflammasome eventually induces an activation of caspase-1 that subsequently cleaves pro-IL-1 β into mature IL-1 β , a potent activator of the NF- κ B signaling pathway, resulting in positive feedback that enhances the immune response^[37]. We therefore examined whether MK-4 represses rotenone-induced caspase-1 activation. In BV2 cells that were treated with rotenone, caspase-1 activation was significantly increased, as indicated by a green fluorescent indicator (FAM-FLICA *in vitro* caspase-1 kits) that can bind to activated caspase-1 (Figure 4A). However, MK-4 decreased the fluorescence intensity of caspase-1 (Figure 4A and 4B) in a dose-dependent manner, further suggesting that MK-4 can suppress rotenone-induced caspase-1 activation.

MK-4 restored the mitochondrial membrane potential damaged by rotenone

Rotenone, a well-known pesticide and inhibitor of mitochondrial complex I^[41, 44], decreases the mitochondrial membrane potential^[45], leading to ROS production^[41, 46]. We therefore examined whether the decreased ROS production by MK-4 is associated with the mitochondrial membrane potential. In BV2 cells treated with rotenone, there was a significant decrease in the mitochondrial membrane potential, which was visualized by TMRM and MitoTracker Green co-staining. However, in the presence of MK-4, the mitochondrial membrane potential was significantly restored (Figure 5A and 5B), suggesting that MK-4 can maintain mitochondrial integrity to decrease the ROS production induced by rotenone.

MK-4 exhibited protective effects against microglia-mediated SH-SY5Y cell death

Activated microglial cells secrete inflammatory molecules that are toxic to neuronal cells^[20, 47, 48]. We previously reported that rotenone induces an activation of BV2 cells, leading to the secretion of inflammatory molecules^[16]. To determine whether MK-4 influences microglia-mediated neuronal cell death, we performed *in vitro* assays to examine the effects of CM from rotenone-treated BV2 cells, with or without MK-4 treatment, on SH-SY5Y cells using PI staining and the MTT assay. The CM collected from BV2 cells that were treated with or without MK-4 was used for the culture of SH-SY5Y cells. The CM from rotenone-treated BV2 cells showed a significant level of toxicity when applied to the SH-SY5Y cells; however, the toxicity of the CM from BV2 cells that were treated with rotenone in combination with MK-4 was obviously reduced (Figure 6A and 6B), suggesting that there are protective effects of MK-4 against microglia-mediated SH-SY5Y cell death.

Discussion

Mitochondrial dysfunction and oxidative stress are tightly associated with the pathogenesis of PD^[6, 49, 50]. Recently, many

studies have indicated that neuroinflammation is involved in DA neuronal degeneration and that an inhibition of neuroinflammation can significantly delay PD progression^[7, 51].

Rotenone induces the activation of NF- κ B, which depends on its nuclear translocation, to transactivate many pro-inflammatory genes^[16]. In the present study, we demonstrate that MK-4 suppresses rotenone-induced activation of NF- κ B and the production of inflammatory factors, including TNF- α , IL-1 β , iNOS and COX-2, by inhibiting the nuclear translocation of NF- κ B in BV2 cells. The phosphorylation of IKK initiates the phosphorylation and degradation of I κ B, leading to the nuclear translocation of NF- κ B^[16]. In our observations, MK-4 inhibits the rotenone-induced phosphorylation of IKK, the degradation of I κ B and the nuclear translocation of the NF- κ B p65 subunit. Furthermore, the rotenone-induced activation of caspase-1 indicates that the activated inflammasome was also decreased in the presence of MK-4. Thus, our results suggest that MK-4 can inhibit rotenone-induced NF- κ B activation and the production of inflammatory factors in microglial cells.

Rotenone-induced NF- κ B activation is dependent upon p38 MAPK to induce ROS production and p38 activation^[16]. Furthermore, ROS was reported to be a second messenger that activates diverse redox-sensitive signaling transduction cascades, including p38 and its associated downstream transcription factors such as NF- κ B and AP-1, thereby regulating the expression of many proinflammatory genes^[52, 53]. Rotenone, as a mitochondrial complex I inhibitor, is reported to decrease the mitochondrial membrane potential, leading to ROS production^[41, 44]. Our study provides evidence that MK-4 not only restores the rotenone-induced decrease in the mitochondrial membrane potential but also inhibits ROS production and p38 activation.

Vitamin K₂ is a fat soluble vitamin that has been suggested to play important roles in maintaining healthy levels of bone and cardiovascular system^[54, 55]. Recently, MK-4, a form of vitamin K₂, was reported to be associated with lower concentrations of inflammatory markers *in vivo* and *in vitro*^[28, 33] and to rescue the deficiency of PD-related genes in the fruit fly (*Drosophila melanogaster*), indicating a protective role of MK-4 in inflammation and PD^[30]. In our study, we reveal that MK-4 suppresses rotenone-induced activation of microglia, most likely via the ROS-p38-NF- κ B pathway. Importantly, MK-4 maintains mitochondrial membrane potential to suppress ROS production, an upstream cellular response to the rotenone insult. The suppression of rotenone-induced microglial activation by MK-4 decreases the production of inflammatory factors, which attenuates inflammatory factor-induced SH-SY5Y cell death. Thus our study provides a mechanistic explanation of how MK-4 functions in anti-inflammation to protect neurons.

Vitamin K includes two natural forms: vitamin K₁ and vitamin K₂, which differ in the lengths of the carbon side chains made of isoprenoid groups of atoms^[27]. Green leafy vegetables have high concentration of vitamin K₁. The bacteria in the colon convert vitamin K₁ into vitamin K₂ to meet our daily needs. Natural vitamin K₂ is also found in bacterially

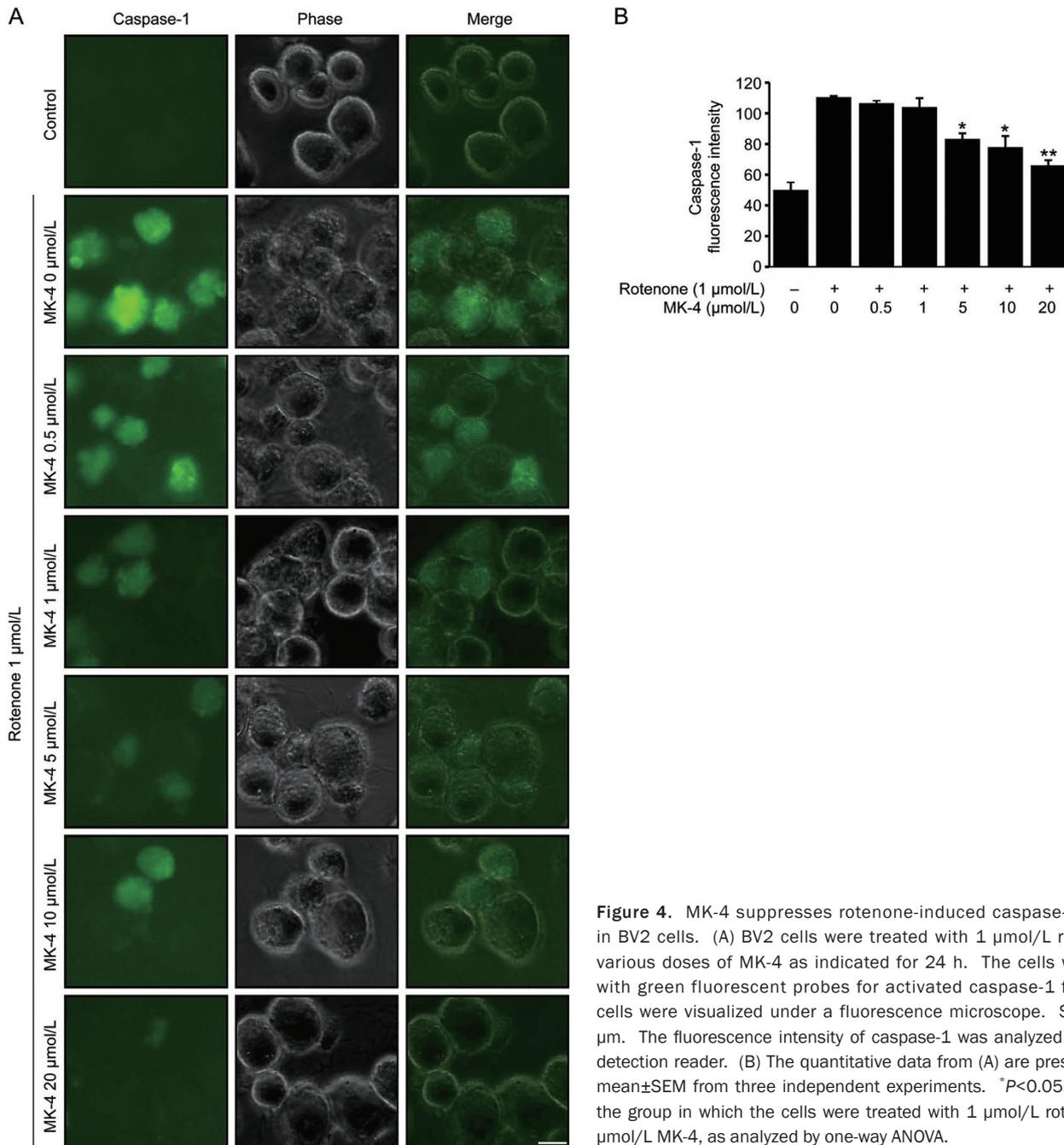


Figure 4. MK-4 suppresses rotenone-induced caspase-1 activation in BV2 cells. (A) BV2 cells were treated with 1 $\mu\text{mol/L}$ rotenone and various doses of MK-4 as indicated for 24 h. The cells were stained with green fluorescent probes for activated caspase-1 for 1 h. The cells were visualized under a fluorescence microscope. Scale bars, 5 μm . The fluorescence intensity of caspase-1 was analyzed with a multi-detection reader. (B) The quantitative data from (A) are presented as the mean \pm SEM from three independent experiments. * $P < 0.05$, ** $P < 0.01$ vs the group in which the cells were treated with 1 $\mu\text{mol/L}$ rotenone and 0 $\mu\text{mol/L}$ MK-4, as analyzed by one-way ANOVA.

fermented foods such as mature cheeses and the traditional Japanese dish natto^[56]. There is no known toxicity associated with a high dose of vitamin K₂, as it is not stored in any significant quantity in the liver. The MK-4 form of vitamin K₂ transfers electrons in the mitochondrial electron transport chain to establish a proton motive force across the membrane in bacteria. It is also sufficient to facilitate mitochondrial electron transport in the fruit fly to rescue impaired mitochondria, similar to ubiquinone (coenzyme Q) and idbenone^[30], two factors that are associated with many illnesses such as PD, inflammation and Friedreich ataxia^[57-60]. Coenzyme Q and idbenone

are similar in structure, including an aromatic ring that may be in either the oxidized (quinone) or reduced (quinol) form but a difference in the length of the lipophilic carbon tail. The completely oxidized form and the completely reduced form of the aromatic ring enable them to perform their functions in the electron transport chain to reduce ROS production. The structure of MK-4 is similar to that of coenzyme Q and idbenone, suggesting that MK-4 may possibly function in the electron transport chain to restore the decreased mitochondrial membrane potential and to suppress ROS production when microglia are exposed to rotenone.

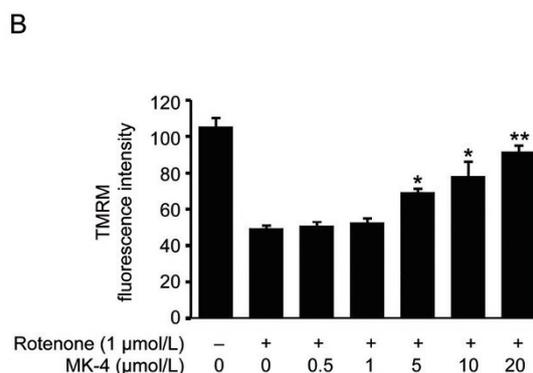
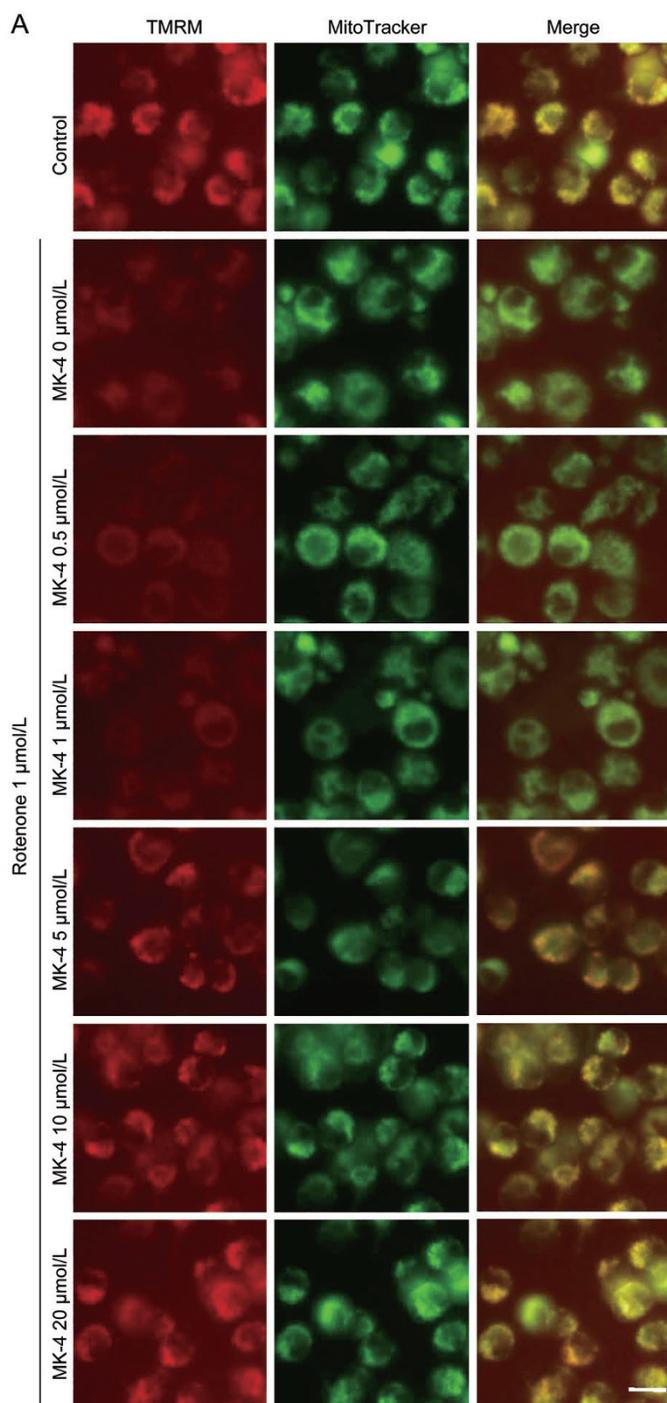


Figure 5. MK-4 restores the mitochondrial membrane potential damaged by rotenone. (A) Various doses of MK-4 and 1 $\mu\text{mol/L}$ rotenone were administered to BV2 cells as indicated for 24 h. The cells were stained with TMRM and MitoTracker Green. TMRM was used to detect mitochondrial membrane potential. MitoTracker Green was used to detect the mitochondria. The cells were visualized under a fluorescence microscope. Scale bars, 5 μm . (B) The fluorescence intensity of TMRM was analyzed with a multi-detection reader. The data are presented as the mean \pm SEM from three independent experiments. * $P < 0.05$, ** $P < 0.01$ vs the group in which the cells were treated with 1 $\mu\text{mol/L}$ rotenone and 0 $\mu\text{mol/L}$ MK-4, as analyzed by one-way ANOVA.

In summary, we have identified that the MK-4 form of vitamin K₂ directly inhibits rotenone-induced NF- κ B activation in BV2 cells. The inhibition of the activated NF- κ B signaling pathway that may be dependent upon p38 MAPK significantly suppresses inflammatory factor production. Furthermore, MK-4 obviously reduces rotenone-induced ROS production and restores the mitochondrial membrane potential. Thus, our study revealed a role for MK-4 in the regulation of microglial activation.

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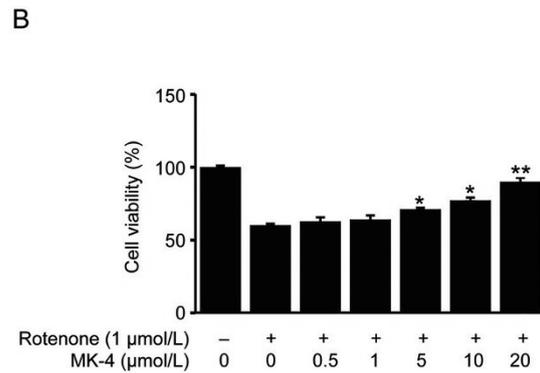
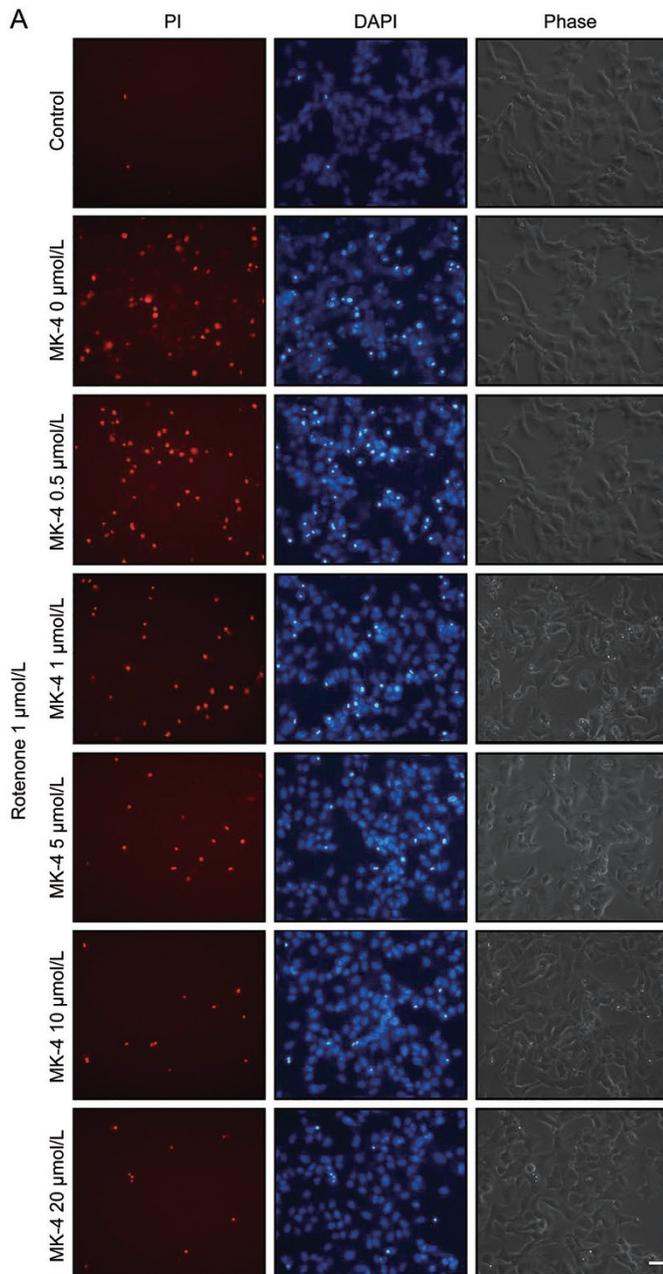


Figure 6. MK-4 rescues rotenone-induced microglia-mediated cell death. The CM was collected from BV2 cells that were treated with rotenone and various doses of MK-4 or without MK-4 for 24 h. The death of SH-SY5Y cells was analyzed using propidium iodide (PI) staining (A) or the MTT assay (B). Scale bars, 20 μm. The data are presented as the mean±SEM from three independent experiments. * $P<0.05$, ** $P<0.01$ vs the group in which the cells were treated with 1 μmol/L rotenone and 0 μmol/L MK-4, as analyzed by one-way ANOVA.

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Author contribution

Dong CHEN and Guang-hui WANG designed the research; Yan-xia YU, Yi-pei LI, Feng GAO, and Dong CHEN performed the research; Qing-song HU and Yan ZHANG contributed new analytical tools and reagents; Dong CHEN and Guang-hui WANG analyzed data; Yan-xia YU drafted the manuscript; Dong CHEN and Guang-hui WANG revised the manuscript

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