PhD Dissertation

Potential role of administration of menaquinone-4 and its chemically related compound for neuroinflammatory modulation

(メナキノン-4 およびその関連化合物の神経性炎症に対する影響)

Wahyu Dwi Saputra

(B9AD1311)

Laboratory of Nutrition

Department of Science of Food Function and Health

Division of Bioscience and Biotechnology for Future Bioindustries

Graduate School of Agricultural Science

Tohoku University

Supervisor :

Prof. Hitoshi Shirakawa

List of Publications

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General Introduction

In the aging society, which is not exclusively found in developed countries, neurodegeneration has become a recent health concern (Kalaria et al., 2008). Neurodegeneration is the condition within the central nervous system (CNS) in which the degenerative process contributes to the progressive loss of neuronal cells and subsequently leads to the CNS-associated cognitive or motoric decline. Alzheimer's and Parkinson's are the primary diseases strongly associated with the neurodegenerative condition called dementia. According to the current report, the number of dementia patients worldwide will be 65.7 million people by 2030. The increasing dementia prevalence is in line with the spike in dementia cost care (Prince et al., 2013). Thus, dementia has had a substantial global socio-economic impact. With this regard, understanding the dementia onset mechanism and its prevention remains elusive.

Generally, the human brain consists of two types of cells: neuron cells and glial cells. Neuron cells make up around 20% of the total brain cells. On the other hand, glial cells that fill the other 80% consist of four types of cells: oligodendrocytes, ependymal cells, astrocytes, and microglia (von Bartheld et al., 2016). The dysfunction of these cells, such as neuroinflammatory conditions, has been reported to contribute to dementia progression. Neuroinflammation is strongly associated with the over-activation of microglial cells (Streit et al., 2004). Microglia is the brain-resident macrophage-like cells that induce innate immunity over CNS. In the physiological environment, microglia are stated as resting cells. Furthermore, these cells can be transformed into different characteristics by specific stimuli. For example, under negative stimuli such as infection, excess oxidative stress, or injury, microglia are transformed to M1 type microglia, which express pro-inflammatory cytokines including interleukin-1β (IL-1β), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), and other chemokines. On the contrary, the anti-inflammatory cytokines induce microglial transformation into M2 type microglia, which produce anti-inflammatory cytokines and repairing substances such as interleukin-4 (IL-4), interleukin-10 (IL-10), and transforming growth factor-β (TGF-β) (Cherry et al., 2014).

With regard to neuroinflammation, microglia are activated due to the occurrence of dangerous stimuli and produce pro-inflammatory cytokines to diminish these threats. Under transient stimulation, the production of cytokines is controllable. However, when the negative stimuli exist for an extended period, microglia undergo cycles of cytokines production

uncontrollably, which further induces neuroinflammation (Mandrekar and Landreth, 2010). The excess levels of cytokines and chemokines due to the neuroinflammation are harmful and potentially cause neuronal cell defects and death, creating neurodegeneration (McGeer and McGeer, 2004). Lipopolysaccharide (LPS) is an endotoxin from the gram-negative bacteria, extensively studied as the potent microglial activator. The intraperitoneal injection of LPS induced neuroinflammation through microglial inflammation in animal-based experiment (Qin et al., 2007; Zhao et al., 2019). In addition, LPS injection-induced the nuclear factor κB (NF-κB) activation is responsible for transactivating pro-inflammatory cytokines and chemokines (Ifuku et al., 2012; Kang et al., 2019). Therefore, NF-κB is the potential target for inflammatory modulation.

In the viewpoint of prevention, daily food consumption containing bioactive compounds is recommended to maintain a healthy status. One promising bioactive compound is vitamin K. Vitamin K has been known for its roles in blood coagulation and bone metabolism. However, several studies recently reported that vitamin K modulates peripheral inflammation, steroidogenesis, and glucose metabolism (Ho et al., 2020; Ito et al., 2011; Ohsaki et al., 2010). In addition, vitamin K exists naturally in two forms: vitamin K_1 (green-leafy vegetables) and vitamin K_2 (fermented product). After consumption, same portion of vitamin K analogs are suggested to convert into menaquinone-4 (MK-4), a subtype of vitamin K_2, in the organ by the UBIAD1 enzyme (Nakagawa et al., 2010). Recent studies reported that MK-4 is distributed in several animal organs, including the brain. Furthermore, MK-4 administration improved LPS-induced inflammation in microglial cell lines, albeit its molecular mechanism was unclear. Moreover, geranylgeraniol (GGOH) is the natural isoprenoid that shares a similar chemical structure with the side chain of MK-4. It is contained in edible grains, rice bran, and vegetable oils (Muraguchi et al., 2011; Ruiz-Aracama et al., 2017). The administration of GGOH has been evaluated to inhibit peripheral inflammation in cell and animal experiments (Giriwono et al., 2019, 2013).

This study mainly evaluated the molecular mechanisms of anti-inflammatory action by MK-4 and GGOH treatments on the LPS-induced inflammation in MG6 mouse microglial-derived cells. In addition, we sought whether the anti-inflammatory properties of MK-4 and GGOH are found not only in the peripheral inflammatory models but also in the CNS inflammatory scenario. We hope that these findings may place preliminary and basic understanding for future experiments in developing MK-4 and GGOH as nutrients and prodrugs for neuroinflammatory modulation.

Aims of Study

The general purpose of this study is to investigate the possible roles of MK-4 and GGOH treatment in the modulation of the neuroinflammatory condition using cell-based experiments with the particular aims are :

1. To characterize the detailed molecular action of MK-4 in the inhibition and recovery of LPS-induced inflammation in MG6 mouse microglial-derived cells.
2. To evaluate the potential of GGOH treatment in ameliorating inflammatory conditions induced by LPS in MG6 mouse microglial-derived cells.
3. To elucidate the further GGOH treatment effect on the inhibition against microglial-mediated neurotoxicity in the HT22 hippocampal neuron cells.

Chapter 1

Literature Review

1.1. Vitamin K

Vitamin K was first discovered in 1929 by Danish biochemist Henrik Dam during his experiment using a low cholesterol diet in chicks. Dam found that a free cholesterol diet caused hemorrhage-like symptoms in chicks that could not be treated using vitamin C. However, the cholesterol diet did not demonstrate similar symptoms. He then proposed vitamin K as nomenclature for 'koagulation' substance, which differs from other fat-soluble vitamins (Ferland, 2012a).

Later, the chemical structure of vitamin K could be characterized. Vitamin K consists of 2 methyl-1,4-naphthoquinone as a ring structure and side-chain, which locates at 3-position of the ring structure. Moreover, scientists found that vitamin K exists in two forms: vitamin K_1 and K_2 in nature. Vitamin K_1 usually is called phylloquinone due to its phytyl side-chain. It is highly distributed in plant products such as green leafy vegetables, grains, or fruits since photosynthesis involves it. On the other hand, vitamin K_2 or menaquinone has a ring structure and prenyl units (4-12) as a side-chain structure (Fig.1a-b). Vitamin K_2 can be found in animal-based products, especially meat and liver, or fermented products like natto and cheese (Shearer and Newman, 2008).

Vitamin K has been widely known to serve as a cofactor for γ–glutamyl carboxylase (GGCX). This enzyme converts specific glutamic acid residues to γ-carboxyglutamic acid (Gla) residues. The Gla itself functions as a calcium-binding protein involved in several biological activities, including blood coagulation, bone metabolism, prevention against calcification, and signal transduction. The growth arrest-specific gene 6 (Gas6) is a vitamin K-dependent protein (VKDP) found throughout the nervous system. It has been reported that Gas6 plays a role in developing the nervous system, including cell proliferation and prevention of apoptosis (Ferland, 2012b). Moreover, a vitamin K antagonist (warfarin) treatment has been demonstrated to cause low sulfatide levels in mice brains (Sundaram et al., 1996). Vitamin K_1 administration was also suggested to associate cognitive function (Chouet et al., 2015). These results hint at the vitamin K function in the nervous system other than its role in the coagulation process.

Recently, vitamin K administration has also been associated with inflammatory modulation. For example, the Framingham Offspring Study found that dietary phylloquinone intake and plasma phylloquinone concentration correlated with the low circulatory inflammatory markers (Shea et al., 2008). Furthermore, vitamin K_2 has been intensively studied as an inflammatory inhibitor and promotes human health (Schwalfenberg, 2017). Moreover, vitamin K_3 (menadione) was also found to attenuate the LPS-induced lung injury either *in vitro or in vivo* (Tanaka et al., 2010). Altogether, these results denoted the potential effect of vitamin K on the inflammatory attenuation, including in the CNS.

1.2. Menaquinone-4

Menaquinone-4 (MK-4) is a subtype of vitamin K_2 with a standard naphthoquinone ring and a side chain with four prenyl units (geranylgeranyl unit) (Fig. 2a). MK-4 is not abundantly contained in food products. Furthermore, intestinal microorganism does not produce it since the MK-4 concentration was not significantly different between normal and germ-free rats (Davidson et al., 1998; Okano et al., 2008; Ronden et al., 1998). However, a previous study reported that MK-4 concentrations are high in several animal organs such as the pancreas, mesenteric fat, testicle, and brain (Shirakawa et al., 2014). Therefore, the consumption of vitamin K was suggested to convert to MK-4 via two routes: the cleavage of vitamin K side chain to menadione in the intestine then prenylation in specific organs or the cleavage and prenylation co-occur in the organ (Okano et al., 2008). Moreover, the UbiA prenyltransferase containing 1 (UBIAD1) enzyme was responsible for those processes. UBIAD1 was ubiquitously present throughout the organs and has both side-chain cleaving and prenylation activity from geranylgeranyl pyrophosphate (the metabolite from the mevalonate pathway) (Nakagawa et al., 2010).

Several studies reported the biological roles of MK-4, which involve steroidogenesis induction (Ito et al., 2011), bile acid modulation in humanized PXR mice (Sultana et al., 2018), inhibition against cancer cell growth and its apoptosis promotion (Duan et al., 2016), and prevention from peripheral inflammation in animal and cell-based experiments (Ohsaki et al., 2010, 2006). Numerous studies also attempted to investigate MK-4 function in the brain since its concentration is high in the nervous system. For example, MK-4 concentration was found to correlate with the levels of sphingomyelin, sulfatides, and gangliosides in the rat brain (Carrié et al., 2004). Another study reported that MK-4 could improve the negative

effect of transient global cerebral ischemia/reperfusion injury in rats via its anti-oxidative and anti-inflammatory properties (Moghadam and Fereidoni, 2020).

In addition, MK-4 was reported to inhibit rotenone-induced inflammation in BV2 microglial cell lines (Yu et al., 2016). Aoyama et al. also demonstrated a similar finding of MK-4 role in the LPS-induced MG6 mouse microglial-derived cells experiment. They found that MK-4 inhibited the production of the pro-inflammatory cytokines via the amelioration of NF-κB activity (Aoyama et al., 2017). However, the detailed molecular mechanism has not been fully clarified. Moreover, the study on the post-treatment effect of MK-4 to recover the inflammatory condition is also still scarce.

1.3. Geranylgeraniol

Geranylgeraniol (GGOH) is a natural isoprenoid belonging to the diterpene groups. Its chemical structure is similar to the side-chain of MK-4 (Fig. 2b). GGOH can be found in vegetable oils such as extra virgin olive oil (0.74 mg/kg), palm oil (3.48 mg/kg), high oleic sunflower oil (2.80 mg/kg) (Purcaro et al., 2016), and edible grains, including rice (0.23 µg/g) (Muraguchi et al., 2011), and rice bran (Kurtys et al., 2018). GGOH is also contained in Brazillian annatto (*Bixa orellana*), ranging from 0.49 to 2.61 g/100 g dry matters (Dequigiovani et al., 2017).

GGOH has been reported for its unique biological function. For example, it has been demonstrated that GGOH treatment enhanced testosterone production in testis-derived cells and plasma testosterone concentration in rats. In addition, GGOH induced the increasing steroid hormones effect via cAMP/PKA signaling, indicating the potential effect of GGOH as a local therapeutic agent (Ho et al., 2018). Moreover, GGOH has been reported in several studies to modulate peripheral inflammatory condition models. For example, GGOH was found to downregulate the pro-inflammatory cytokines expressions in aminobisphosphonate-induced RAW264.7 cells and LPS-treated THP-1 human macrophagic cells and rats (Giriwono et al., 2019, 2013; Marcuzzi et al., 2010). Furthermore, GGOH was required to maintain endotoxic tolerance in repeated treatment of LPS on macrophage-like cells (Kim et al., 2013). Specifically, GGOH was reported to target the NF-κB activation in hepatocarcinogenic rats (de Moura Espíndola et al., 2005).

In the context of the nervous system, consistent GGOH production was known to support the long-term potentiation in hippocampal slice experiments and promote learning in

aminals (Kotti et al., 2006). Furthermore, the isoprenoid production was also known in association with the low levels of amyloid-β (Cole et al., 2005). Moreover, the statin administration reduced the concentration of isoprenoids and contributed to neuron cell defects, while GGPP administration reversed this fashion (Tanaka et al., 2000). Recently, the exogenous administration of GGOH was reported to induce protein isoprenylation in mammalian cells. It indicated the possibility that free GGOH could be converted to GGPP (Crick et al., 1997). Another study reported that GGOH is converted to geranylgeranoic acid (GGAC) and shows an anti-carcinogenic effect (Muraguchi et al., 2011). Nevertheless, exogenous GGOH administration shows promising therapeutic effects.

1.4. Microglia

Microglia were discovered and named by Spanish neuroanatomist Pio del Rio-Hortega (1882-1945). They originate from the pool of primitive macrophages which enter the brain during embryonic development and persistently exist throughout adulthood (Kettenmann et al., 2011). Therefore, microglia belong to the immune cell family. Given that reason, microglia may possess distinct functions and characteristics. During post-natal development, microglia have an activated phenotype and high proliferation, which will switch to the steady-state phenotype. Initially, microglia support the development of neuron and oligodendrocyte cells via the production of insulin-like growth factor 1 (Prinz et al., 2019).

In the adult brain, microglia affirm resting conditions, morphologically indicated by highly ramified branches. The ramified microglia are responsible for surveying the brain environment for any possible condition that requires further action. In this state, microglia also produce nerve growth and fibroblast growth factors to maintain neuronal cell viability and circuit formation. Moreover, ramified microglia also play roles in the phagocytosis of debris and defected neuronal cells. Thereby, they maintain nervous system homeostasis (Subhramanyam et al., 2019).

Due to specific stimulations such as infection, brain injury, or neurodegeneration, the ramified cells can be converted to ameboid-type cells with short dendritic arbors and enlarged soma. Several substances have been reported as the strong microglial inducers, including LPS, interferon-gamma (IFN-γ), amyloid-β, α-synuclein, and TNF-α (Subhramanyam et al., 2019). Upon the stimulation, microglia produce a broad spectrum of cytokines and chemokines such as IL-1β, IL-6, and TNF-α, along with the increasing levels of nitric oxide (NO) and reactive

oxygen species (ROS). Traditionally, this condition was classified as the M1-phased microglia. The aged microglia are more responsive and susceptible to dangerous stimulation. The chronic production of cytokines and chemokines then contributes to the neuroinflammatory conditions (Wolf et al., 2017).

Alternatively, microglia can also be transformed into another phenotype by interleukin-4 (IL-4) and interleukin-13 (IL-13), termed M2-phase (Cherry et al., 2014). The M2 microglia are characterized by the production of anti-inflammatory cytokines such as interleukin-10 (IL-10) and transforming growth factor-β (TGF-β) and wound healing substances such as arginase-1 (ARG-1) and found in inflammatory zone-1 (FIZZ-1) (Raes et al., 2002). However, the dichotomy of M1 and M2 cell phenotypes is still debatable. Indeed, microglia act in benefit and negative modes. The continuous disruption in the balance between those two modes will initiate and augment neuropathological conditions. In addition, the inflammatory state of microglia has been suggested to trigger synapsis loss, injure neuron cells, and spread the tau pathology, which develops Alzheimer's disease (AD) onset (Hansen et al., 2017).

1.5. Neuroinflammation

Inflammation is a strategy of cells or tissues to react to injury and control the stimulus. Under the temporal condition, inflammation is proposed as a beneficial reaction. However, when excessive stimulus stability occurs, inflammation turns to tissue damage and disease contributor. It targets the injury site and the neighboring tissue resulting in extended and systemic reactions. Neuroinflammation can be induced directly by negative stimuli such as infection and injury or peripheral inflammation (Lyman et al., 2014).

Previously, the cytokines produced in peripheral inflammation were assumed to be large molecules that could not go through blood-brain barriers (BBB). However, recent studies reported that the active transport system in BBB facilitates the infiltration of TNF-α and IL-cytokines to the mouse brain (Gutierrez et al., 1993). Moreover, higher levels of IL-1β, IL-6, and COX-2 were suggested to affect the integrity of BBB, allowing it to be more permeable (Terrando et al., 2011). Once the cytokine or infection infiltrates the brain, they stimulate the activation of microglia. Furthermore, either infiltrated cytokines or endogenous cytokines produced by microglia can induce further microglia activation via the induction of their receptors, such as IL-receptor and TNF-receptors (Riazi et al., 2008). In addition,

intraperitoneal injection of LPS has enhanced cytokine levels in animal brains and promoted cognitive impairment (Arai et al., 2004; Biesmans et al., 2013).

Indeed, neuroinflammation has a strong manifest in the progression of neurodegenerative diseases. IL-1β, TNF-α, and COX-2 have been associated with the synaptic defect (Cunningham et al., 1996; Stark and Bazan, 2011). IL-6 and TNF-α inhibited the differentiation of neural progenitor cells leading to the disfunction of neurogenesis (Liu et al., 2005). Moreover, the chronic production of pro-inflammatory cytokines was found in association with the synthesis of amyloid precursor protein (APP) which results in the production of amyloid β, the hallmark of AD (Ghosal et al., 2009). NF-κB activation by TNF-α was also reported to contribute to APP cleaving enzyme-1 (BACE-1) (Chen et al., 2012). Therefore, targeting microglia in neuroinflammation is a promising strategy to prevent or slow neuropathological development.

1.6. The NF-κB signaling pathway

NF-κB is the inducible transcription factor regulating many genes involved in immune and inflammatory reactions. In mammalian cells, the NF-κB family is composed of five proteins, including p65 (RelA), RelB, c-Rel, p105/p50 (NF-κB1), and p100/52 (NF-κB2). These proteins are associated with each other as homo or heterodimers, which bind to the DNA site, κB enhancer, to mediate distinct transcriptional activities (Oeckinghaus and Ghosh, 2009). In addition, several stimuli initiate NF-κB activity through the activation of membrane receptors, such as pattern recognition receptors (PRRs), TNF-receptor (TNFR), cytokine receptors as well as T cell and B cell receptors (Liu et al., 2017).

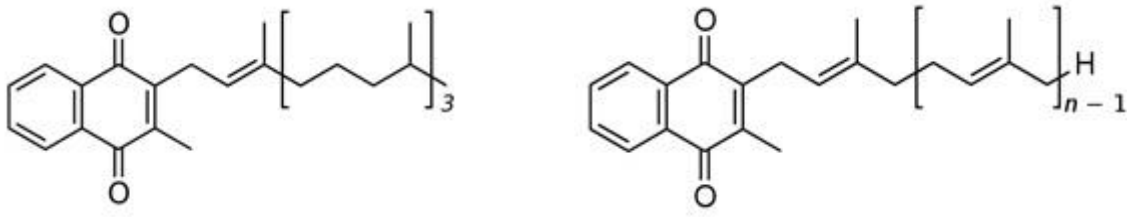
LPS has been widely known as NF-κB solid inducer. It induces the NF-κB via the activation of TLR4, which is located in the cell membranes. The activation of TLR4 triggered the activation of IRAK1 and recruitment of NEMO and phosphorylation of TRAF6, which in turn induced the activation of TAK1. NEMO recruitment in IRAK1-TRAF6 will allow the TAK1 to induce the phosphorylation of IKKα/β (Windheim et al., 2008). IKKα/β is the kinases for the phosphorylation of IκBα, the inhibitor protein which sequesters NF-κB in the cytoplasmic compartment. The phosphorylation of IκBα triggers its proteasomal degradation leading to the free of NF-κB heterodimers to translocate into the nuclear part, binds to its recognizing site, and induce the pro-inflammatory cytokines transactivation (Oeckinghaus and Ghosh, 2009) (Fig. 3).

In the frame of controlled reaction, NF-κB would undergo post-translational modification to limit the detrimental inflammatory effect. The post-translational regulation, called negative feedback regulator, comprises two-main categories, the signal-specific and gene-specific regulators. The signal-specific regulator limits the NF-κB activation by inhibiting the signal transduction pathway. This includes the production of A20, ST2, IL-1R-associated kinase M (IRAK-M), and suppressor of cytokine signaling (SOCS) family proteins. The SOCS1 was suggested to facilitate the nuclear NF-κB p65 proteasomal degradation. In addition, the concept of resynthesized IκBα, which then exports nuclear NF-κB p65, is also included in this category. In the second category, the negative regulator works on the transcriptional activity to repress the production of inflammatory genes. The IκB-NS and BCL-3 are the two proteins that mediate the exchange of active NF-κB dimers to the inactive counterparts in target gene promoters. IκB-NS controls the transcription of IL-6, IL-18, while BCL-3 limits the transcription of TNF-α and IL-1β (Medzhitov and Horng, 2009).

1.7. Microglial-mediated Neurotoxicity

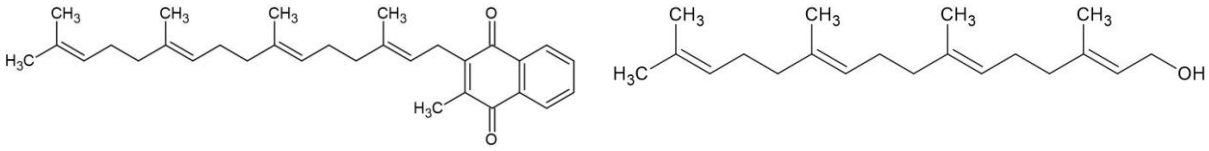
We mentioned above that excessive microglial inflammation induces neurodegeneration via neuronal cell death. The previous review proposed the possible way microglia mediates neurotoxicity: first, the classical inflammatory mechanism which produces harmful pro-inflammatory factors; second, the overactivation of microglia in response to the neuronal damages. The defect neurons also secrete microglial activators such as laminin, MMP3, α-synuclein, and neuromelanin. These reactions may undergo perpetuating cycle, which further causes progressive neuron death (Block et al., 2007).

Microglia-produced pro-inflammatory cytokines were associated with neuronal defects via distinct pathways. For example, TNF-α binds to TNFR and recruits FADD and Caspase-8. This complex further induces programmed cell death or apoptosis (Micheau and Tschopp, 2003). Furthermore, IL-1β from microglia was reported to trigger mitochondrial apoptosis in neuronal precursor cells and induce cell cycle arrest via p53 activation (Guadagno et al., 2015). On the other hand, NO synthesized from iNOS disrupted the neuron respiration leading to glutamate release. The glutamate, in turn, activated NMDA receptors and induced excitotoxic death (Bal-Price and Brown, 2001). The neuronal excitotoxicity had also occurred when the concentration of COX-2 was too high, causing overproduction of prostaglandin (Stark and Bazan, 2011).



(a) (b)

Fig. 1. Chemical structures of the vitamin K vitamers: phylloquinone (a) and menaquinone (b)



(a) (b)

Fig. 2. Chemical structures of the menaquinone-4 (MK-4) (a) and geranylgeraniol (GGOH) (b)

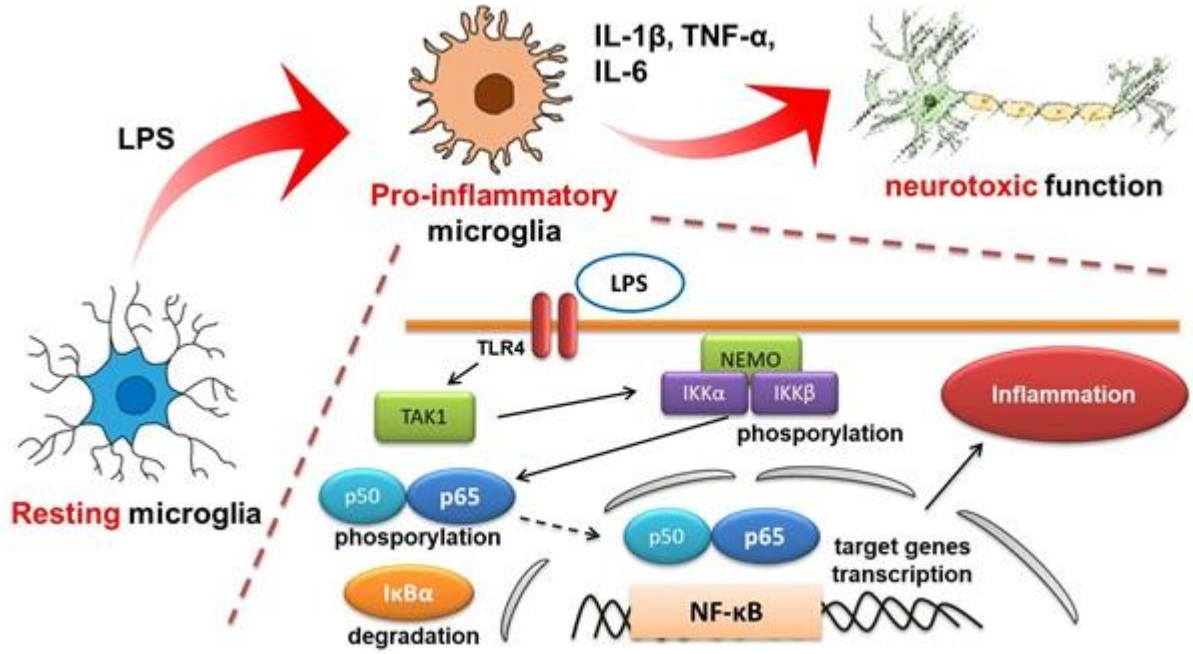


Fig. 3. NF-κB signaling pathway during LPS-induced inflammation. LPS activates TLR4 which is responsible for the subsequent molecular transduction. The TAK1, IKKα/β, and p65 subunit of NF-κB phosphorylations were followed by the p65 nuclear translocation and transactivation of inflammatory cytokines.

Chapter 2

MK-4 inhibited the NF-κB nuclear translocation and alleviated the inflammatory mRNA expression after LPS administration in MG6 mouse microglial-derived cells.

2.1. Introduction

Menaquinone-4 (MK-4), a subtype of vitamin K_2, has been reported for its unique function to ameliorate peripheral inflammatory conditions in cell and animal-based experiments (Ohsaki et al., 2010, 2006). Moreover, MK-4 treatment was also found to inhibit the rotenone-induced inflammation in BV2 microglial cells (Yu et al., 2016). Furthermore, Aoyama et al. demonstrated that MK-4 pre-treatment effectively down-regulated the pro-inflammatory cytokine expressions in LPS-induced MG6 mouse microglia-derived cells using another cell line type. They also observed that MK-4 targetted NF-κB activation by inhibiting the phosphorylation in the NF-κB p65 subunit (Aoyama et al., 2017). In addition, LPS has been widely known for its induction on the microglial activity, including NF-κB signalling (Kang et al., 2019).

The functional mechanism underlying NF-κB activation is the proteasomal degradation of inhibitor κB (IκBα) protein, which subsequently liberate the NF-κB in the cytoplasmic. After its relieve, NF-κB will translocate to the nucleus and bind to its recognizing site to initiate the transactivation of pro-inflammatory genes. Since Aoyama et al. found the inhibitory effect of MK-4 against the NF-κB p65 phosphorylation, we focused to study the more detailed molecular action of MK-4 effect on the NF-κB p65 dynamics. First, we evaluated the LPS injection effect on the pro-inflammatory mRNA expression in mice brains. Thereafter, we utilized fluorescence imaging to visualize the NF-κB p65 distribution in MG6 cells under the influence of MK-4 and LPS. Moreover, we confirmed the imaging analysis by measuring and comparing the NF-κB protein levels in the cytoplasmic and nuclear fraction of MG6 cells.

In addition, after the activation, NF-κB is subjected to post-translational modification, which aims to limit and recover the inflammatory condition. Since MK-4 has been suggested to prevent inflammation in the pre-treatment scenario, we evaluated whether MK-4 post-treatment could recover the inflammatory condition initiated by LPS administration in MG6 cells. Therefore, we analyzed the pro-inflammatory cytokine mRNAs expressions and NF-κB dynamics in MG6 cells administered first using LPS followed by MK-4 treatment.

2.2. Materials and Methods

2.2.1. Materials

MK-4 was provided by Nishin Pharma Inc. (Tokyo, Japan). As the stock reagents, it was dissolved in 99% ethanol (Wako Pure Chemicals, Osaka, Japan) at 10 mM concentration. Furthermore, MK-4 volume was adjusted to 0.1% (v/v) in cell media in the experiment. AIN-93M standard diet components were purchased from Wako and Oriental Yeast Co., Ltd. (Tokyo, Japan)

2.2.2. Cell Culture

c-*Myc*-immortalized MG6 mouse microglial cells (RCB2403) were provided by RIKEN Cell Bank (Tsukuba, Japan) (Takenouchi et al., 2011, 2005). Cells were seeded in DMEM high glucose media (Sigma-Aldrich) containing 10% fetal bovine serum (Biowest, Nuallie, France), penicillin (100 units/mL), streptomycin (100 µg/mL), 10 µg/mL of human recombinant insulin (Gibco Thermo Fisher Scientific, Waltham, MA, USA), and 100 µM β-mercaptoethanol (Wako). Cells were maintained in a 37 ºC incubator with 5% CO_2 and monitored using an inverted microscope. After reaching 80% of confluency, cells were passaged into fresh media (Saputra et al., 2019).

2.2.3. Animal and Treatment

Male C57BL/6J mice (11-12 weeks old) were purchased from CLEA Japan Inc. (Shizuoka, Japan) and maintained in plastic cages (2 mice per cage) under controlled temperature (23 ± 2ºC), 50% ± 10% humidity, and 12:12-h automatic dark-light cycle. After 1-week acclimatization, the mice were grouped into the control and LPS groups. All mice were fed with AIN-93M standard diet for two weeks. In the third week, mice were intraperitoneally injected with saline 0.9% for the control group or LPS (0.5 mg/kg) for the LPS group for 5 consecutive days. On the final day, all mice were sacrificed, and brain cortex tissues were quickly isolated, immersed in RNA later solution, and stored at -80ºC for RNA analysis.

2.2.4. Fluorescence Microscopy

The MG6 cells were treated with MK-4 (10 µM) for 24 h, followed by LPS (1 ng/mL) administration for 30 min. The cell media were then removed, and cells were fixed using 4% paraformaldehyde in PBS for 15 min. After that, cells were permeabilized with 0.25 % Triton

X-100/PBS and blocked using 4% fetal bovine serum (Gibco) in PBS for 1 h at room temperature. The blocked cells were then subjected to primary antibody incubation overnight at 4 ºC using an anti-p65 antibody (1:1000) (Cell Signalling Technology, Danvers, MA, USA). The secondary antibody incubation was performed on the following day using Alexa Fluor 555 goat anti-rabbit IgG (Invitrogen, Waltham, MA, USA) for 1 h, followed by the nuclear staining using Hoechst 33258 at 1µg/mL concentration. The fluorescence visualization was done using Olympus IX81 (Olympus, Tokyo, Japan) fluorescence microscope (Saputra et al., 2019).

2.2.5. Cytoplasmic-Nuclear Fractionation

The cell treatment procedures are indicated in the figure captions. After the treatments, cell media were removed, washed using cold 1X PBS, and pelleted by centrifugation at 3000 rpm for 3 min. The cytoplasmic buffer containing 320 mM sucrose, 3 mM $CaCl_2$, 0.1 mM EDTA, 1 mM dithiothreitol (DTT), 2 mM $MgCl_2$, and 0.5% NP-40, supplemented with proteinase and phosphatase inhibitors (Roche Applied Science, Mannheim, Germany) was then added and incubated for 20 min. On the final incubation period, the mixtures were vortexed vigorously and separated using centrifugation at 600 *g*, 15 min. The supernatants were transferred to prechilled microtubes as cytoplasmic fractions. On the other hand, pellets were then subjected to nuclear buffer containing 20 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 420 mM NaCl, 1 mM DTT, 0.3% NP-40, 25% glycerol, and tablets of proteinase and phosphatase inhibitors. The mixtures were vortexed every 10 min for a total 40 min, followed by centrifugation at 16000 *g*, 10 min. The final supernatants were collected on prechilled microtubes as nuclear fractions. All the procedures were done on ice to avoid protein denaturation. All the fractions were kept at -80 ºC until used in western blot analysis (Saputra et al., 2021).

2.2.6. Whole Cell Protein Extraction and Western Blot Analysis

After the treatments as indicated in each figure caption, whole-cell proteins were extracted using lysis buffer containing 50 mM Tris-HCl pH 7.5, 5 mM EDTA, 150 mM NaCl, 0.1% SDS, and 1% NP-40 in the presence of proteinase and phosphatase inhibitors. Supernatants were collected after centrifugation at 13000 rpm for 15 min. The protein amount was measured using the Lowry method to make equal protein volume and denatured using a 3X SDS loading buffer.

The western samples were subjected to an electrophoresis separation system using 10-20% SDS-polyacrylamide pre-stacked gel (Wako Pure Chemicals). Thereafter, the separated protein was transferred to PVDF membrane (Millipore, Billerica, MA, USA) in a semi-dry transfer system (Bio-Rad, Hercules, CA, USA) and blocked using 3% bovine serum albumin (BSA) (Sigma-Aldrich) in TBS-Tween (TBST). After blocking, the membrane was incubated overnight at 4 ºC using a primary antibody as listed in Table 1.1. The primary antibodies dilution was adjusted at a 1:1000 ratio. The following day, the membrane was washed in TBST and incubated with a secondary antibody in a 1:5000 dilution ratio. For loading control, the membrane was stained using α-tubulin for general protein or lamin for nuclear protein. The protein bands were visualized using ChemiDoc Imaging System (Bio-Rad, Richmond, CA, USA). Moreover, the densitometric band evaluation was measured using Image Lab 6.1. (Bio-Rad) (Saputra et al., 2021).

2.2.7. Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) Analysis

MG6 cells were treated as indicated in the figure caption. First, RNA extraction from cells or brain cortex tissues was performed using a commercially available reagent, ISOGEN (Nippon Gene, Tokyo, Japan). After that, the RNA amount and 260/280 absorbance were measured using nanodrop analysis (Thermo Fisher Scientific). Next, one microgram of RNA was used to synthesize the cDNA by denaturing with oligo-dT primers and dNTPs, followed by mixing with RT buffer, DTT, RNase OUT RNase inhibitor, and SuperScript III reverse transcriptase. cDNA aliquots were then amplified using gene-specific primers as listed in Table 1.2. and SYBR Premix Ex Taq (Takara Bio, Otsu, Japan) in CFX Connect Real-Time PCR Detection System (Bio-Rad). Finally, the mRNA expressions were normalized to the eukaryotic elongation factor 1α1 (*Eef1a1*) as a house-keeping gene (Saputra et al., 2021).

2.2.8. Statistical Analysis

The results are presented as mean ± standard error of the mean. Data were analyzed using one-way analysis of variance (ANOVA) in SigmaPlot version 12.5 (San Jose, CA, USA). Multiple comparisons were performed using the Tukey-Kramer test. The statistical differences were recognized at $p < 0.05$.

2.3. Results

2.3.1. The up-regulation of cortex pro-inflammatory cytokines after LPS injection

In this experiment, we observed that LPS intraperitoneal injection effectively induced the mRNA expressions of *Il-1β* and *Tnf-α* in the mice brain cortex, as shown in Fig. 4. It suggested that LPS is the potent inducer for neuroinflammation. Therefore, we mimicked this event in the subsequent experiments by administering LPS in MG6 mouse microglial-derived cells.

2.3.1. MK-4 pre-treatment inhibited the NF-κB p65 nuclear translocation

We checked the MK-4 effect on the NF-κB p65 subunit nuclear translocation. We found that under normal situations, NF-κB p65 was predominantly located in the cytoplasmic area, as shown in Fig. 5 (control group). LPS administration markedly induces the nuclear translocation of NF-κB p65 indicated by the intense p65 staining in the nuclear area of treated MG6 cells. However, MK-4 treatment was observed to reduce the intensity of nuclear p65 staining suggested that MK-4 effectively inhibited LPS-induced NF-κB p65 translocation. Our visual data was further confirmed using cytoplasmic-nuclear fractions analysis in western blot, as shown in Fig. 6a-b. We detected that the nuclear NF-κB p65 level under LPS stimulation was the highest among the treated groups, and MK-4 pre-treatment significantly inhibited that. These results hint that MK-4 functions in NF-κB p65 to suppress its phosphorylation and interfere with its nuclear transfer.

2.3.2. The reduction of pro-inflammatory cytokine mRNA expression after LPS depletion from MG6 cells

We confirmed that MK-4 has a preventive effect against the LPS-induced inflammation in MG6 cells. We then attempted to evaluate the post-treatment effect of MK-4 in similar models. We initially initiated the inflammation by providing LPS in the first row. After 3 h of LPS incubation, we discarded the cell media containing LPS and washed the cells using PBS two times to diminish the LPS effect. Thereafter, we administered MK-4 to MG6 cells for 2 h and evaluated the pro-inflammatory cytokine expressions. Interestingly, either with or without the post-treatment of MK-4, the pro-inflammatory cytokines mRNA expressions including *Il-1β*, *Tnf-α*, and *Cox-2* were dramatically reduced (Fig. 7a-d). It denoted that after LPS deletion, cells may return to basal condition although without any

post-treatment. Hence, this scenario may not be suitable to evaluate the post-treatment of MK-4.

2.3.3. MK-4 post-treatment reduced the pro-inflammatory cytokines initiated by LPS

Here, we induced the inflammation in MG6 cells by administering the LPS for 3 h, and without any depletion, we followed to treat the activated cells using MK-4 for another 2 h. We observed that the *Il-1β*, *Il-6*, *Tnf-α*, and *Cox-2* mRNA expressions were steadily enhanced in MG6 cells without any MK-4 post-treatment. However, the post-treatments of MK-4 dose-dependently showed a negative effect against the LPS influence, indicated by the reducing level of those mRNA expressions (Fig. 8a-d). In this scenario, we demonstrated that the post-treatment effect of MK-4 could recover the inflammatory condition initiated by LPS in MG6 cells. Moreover, MK-4 showed an up-regulation effect on the negative inflammatory regulator mRNA expressions, including suppressing cytokine signaling 1 (*Socs1*) and *Bcl-3* (Fig. 8e-f).

2.3.4. MK-4 post-treatment tended to reduce the nuclear NF-κB level

We mentioned above that the NF-κB activation regulates the pro-inflammatory cytokines via binding to recognizing sites in cell nuclei. Since we found that post-treatment of MK-4 reduced the pro-inflammatory cytokines mRNA expressions, we then checked the nuclear NF-κB p65 dynamic. As shown in Fig. 9a, the nuclear NF-κB p65 levels were reduced in time-dependent manners, suggesting a linear effect as found in pro-inflammatory mRNA analysis. When we post-treated MK-4 to the activated MG6 cells without any LPS depletion, we found that MK-4 tended to reduce the nuclear NF-κB p65 levels (Fig. 9b). This result shows that MK-4 may inhibit the translocation of NF-κB p65 and potent from affecting its post-translational modification, which contributes to its post-treatment effect.

2.4. Discussion

We found that MK-4 pre-treatment effectively inhibited the NF-κB p65 subunit nuclear translocation. Our finding corresponds with the previous experiment that observed the inhibitory of NF-κB p65 translocation by MK-4 in BV2 microglial cells (Yu et al., 2016). By inhibiting the NF-κB p65 translocation, the further binding of NF-κB to its recognizing site could be prevented. In consequence, the transactivation of pro-inflammatory cytokines could also be modulated. The further mechanism on how MK-4 inhibited the NF-κB was unclear. MK-4 ameliorated the IKK phosphorylation in macrophage-like cells, which

contributes to the inhibition against subsequent signaling (Ohsaki et al., 2010). However, Aoyama et al. did not find a similar effect in MG6 cells. Instead, they found that simultaneous treatment of MK-4 in MG6 maintained the IκBα protein level (Aoyama et al., 2017). Hence, we assumed that NF-κB p65 translocation inhibition was achieved via the retaining of IκBα, which sequesters the NF-κB in cytoplasmic. One possible mechanism is that MK-4 binds to the subunit of 26S proteasome and blocks the IκBα degradation (Gao et al., 2000). Further evaluations are worth considering to elucidate this hypothesis.

In the first post-treatment scenario, we administered MG6 using LPS then washed out the LPS from cells. We observed a significant reduction of pro-inflammatory mRNA expressions, either with or without MK-4 post-treatment. Here, we understood that cells progressively underwent self-termination against inflammatory reaction once the stimuli were eliminated. We detected that 1 h after LPS depletion, the NF-κB p65 was still in the nuclear part at a high level. However, after 2 h depletion, the nuclear NF-κB p65 was started to reduce (Fig. 7a). We speculated that this fashion contributes to dramatically reducing pro-inflammatory mRNA expressions after 2 h LPS washing out, even without any MK-4 treatment.

MK-4 post-treatment significantly reduced the pro-inflammatory mRNA expressions when the LPS was not diminished from cells. It suggested the promotion of negative feedback effect by MK-4. The possible mechanism is that MK-4 induces the export of NF-κB p65 from nuclear to cytoplasmic (Medzhitov and Horng, 2009). However, even though there was a tendency in our experiment, we did not detect a statistical difference considering that matters. Another plausible mechanism is that MK-4 promotes the negative transcriptional feedback, either by inducing gene-specific regulators or triggering mRNA degradation (Kuwata et al., 2006). It was suggested that post-treatment of MK-4 could up-regulate the negative feedback regulator mRNA expressions, including *Socs1* and *Bcl-3*.

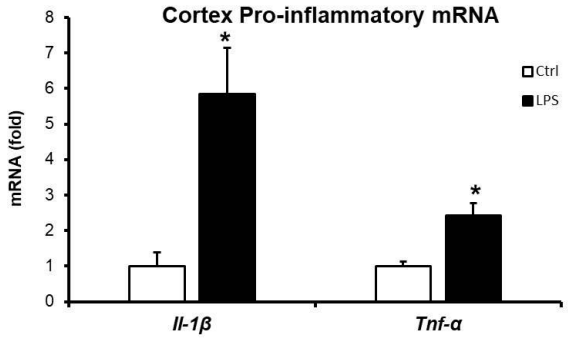


Fig. 4. The induction of *Il-1β* and *Tnf-α* mRNA expression in mice brain cortex by LPS injection. Mice were intraperitoneally injected with saline 0.9% (control) or LPS (0.5 mg/kg) (LPS) for 5 consecutive days. The mRNAs were analyzed by qRT-PCR, normalized to the levels of *Eef1a1* (elongation factor), and expressed as a fold of the control group. Data are presented as the mean ± S.E.M, $n = 6$; * $p < 0.05$ vs. control (saline-injected group).

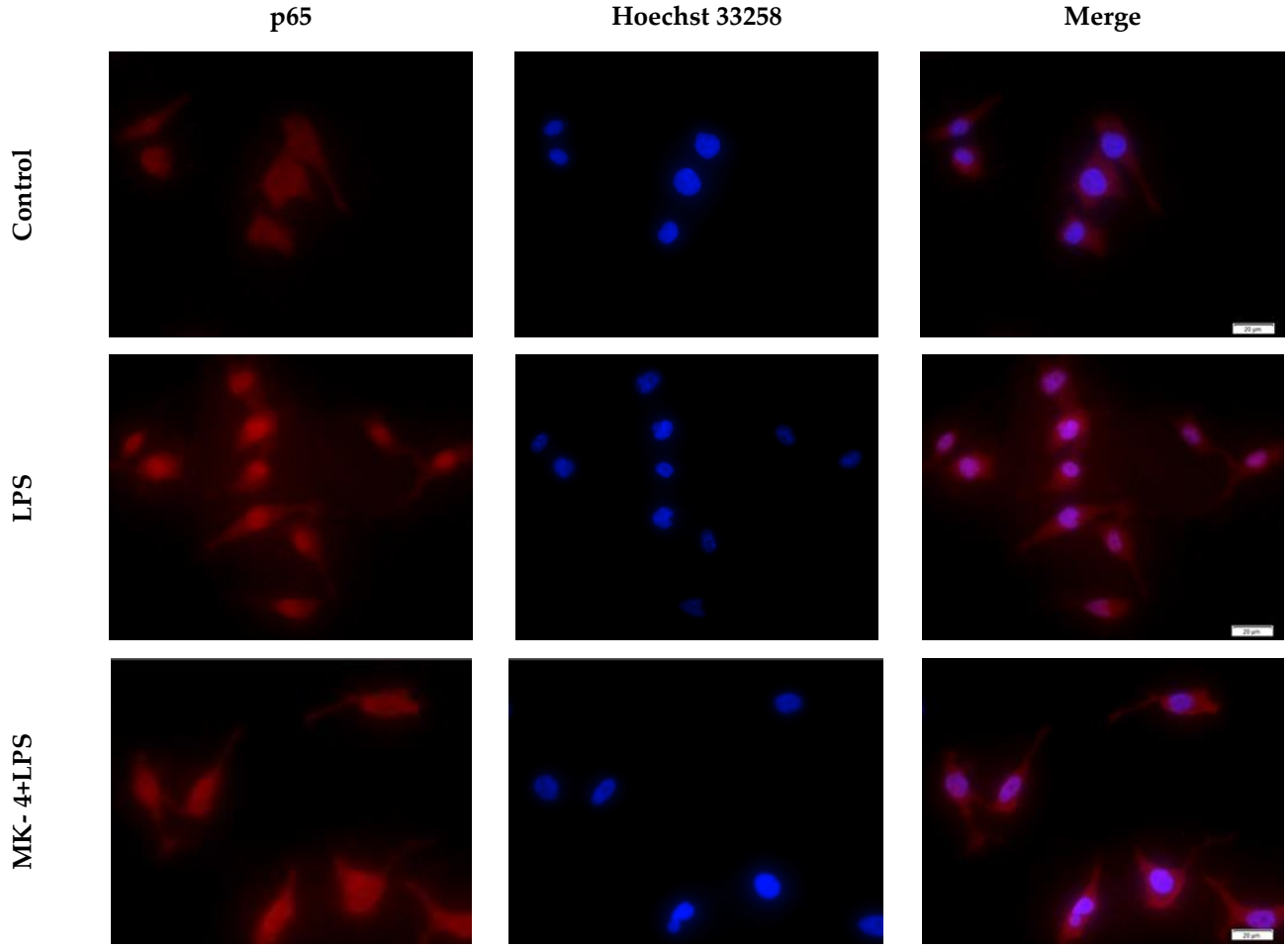
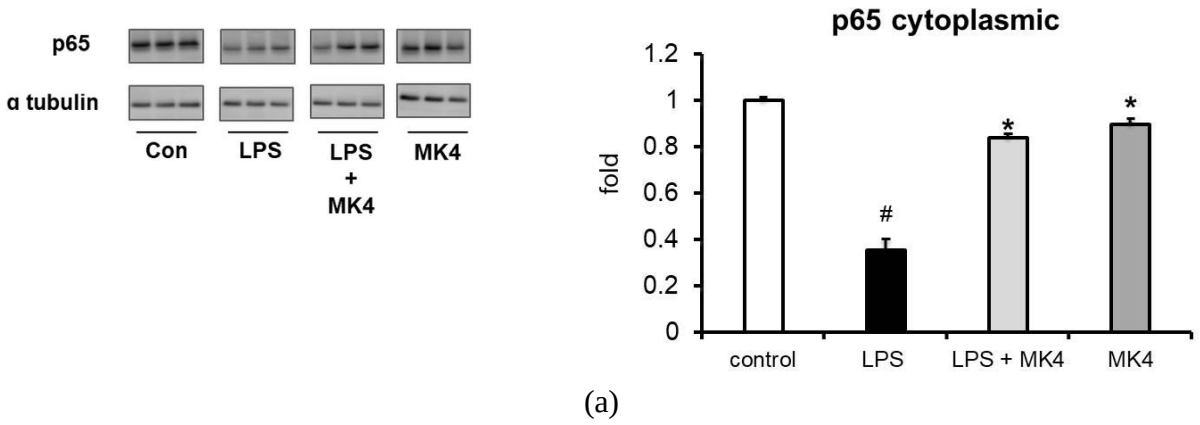
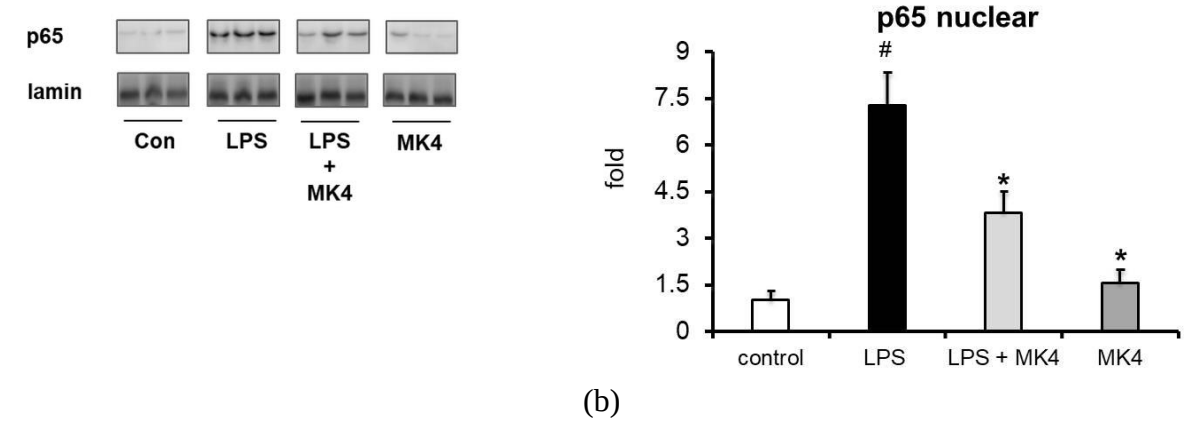


Fig. 5. The inhibition of LPS-induced NF-κB p65 subunit nuclear localization by MK-4. MG6 cells were administered with MK-4 (10 μM) for 24 h then followed by the treatment of LPS (1 ng/ml) for 30 min. The cells were stained with anti-p65 (red) antibodies. After that, the cells were visualized under a fluorescence microscope. The nuclei were stained with Hoechst 33258 (1 μg/ml) (blue). Scale bars, 20 μm). (cited from Saputra et al., Int. J. Mol. Sci. 2019, 20, 2317).



(a)



(b)

Fig. 6. The blocking of NF-κB p65 nuclear translocation by MK-4 administration. MG6 cells were fractionated into cytoplasmic (a) and nuclear fractions (b) and analyzed using western blot. Data are presented as mean ± SE (n = 3), normalized to the α-tubulin (cytoplasmic) or lamin (nuclear) levels and expressed as a fold of the control cells values. n = 3; # $p < 0.05$ vs. untreated control; * $p < 0.05$ vs. LPS-only treated group. (cited from Saputra et al., Int. J. Mol. Sci. 2019, 20, 2317).

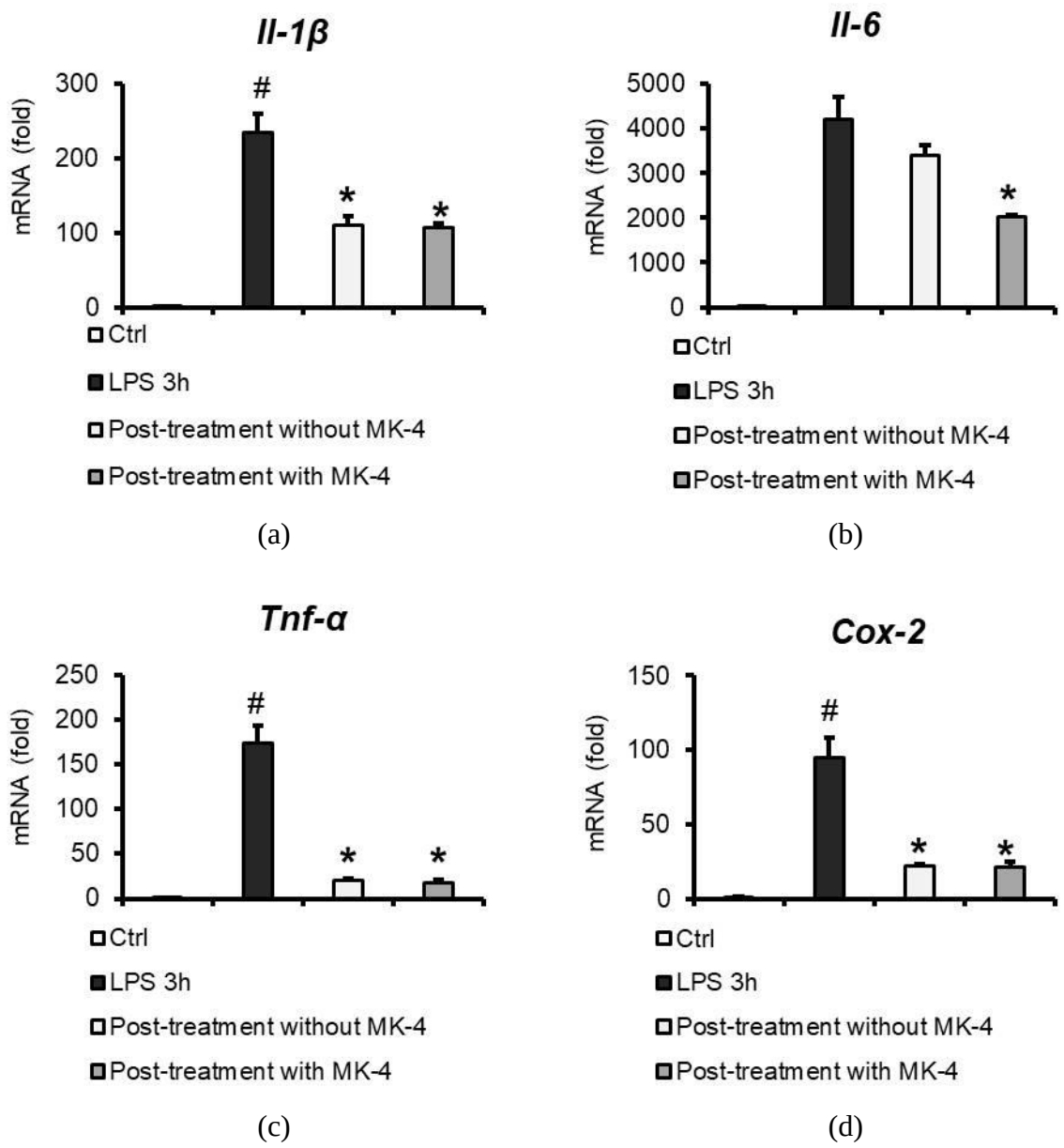


Fig. 7. The cytokines mRNA expressions were dramatically reduced either with or without MK-4 post-administration. MG6 cells were first stimulated by LPS (10 ng/mL) for 3 h, the medium was then discarded and washed two times with PBS and replaced with a new medium containing with or without MK-4 (10 μM) for 2 h. The mRNAs were analyzed by qRT-PCR, normalized to the levels of Eef1a1 (elongation factor), and expressed as a fold of the control group. Data are presented as the mean ± S.E.M, n = 3; # $p < 0.05$ vs. untreated control; * $p < 0.05$ vs. LPS-only treated group.

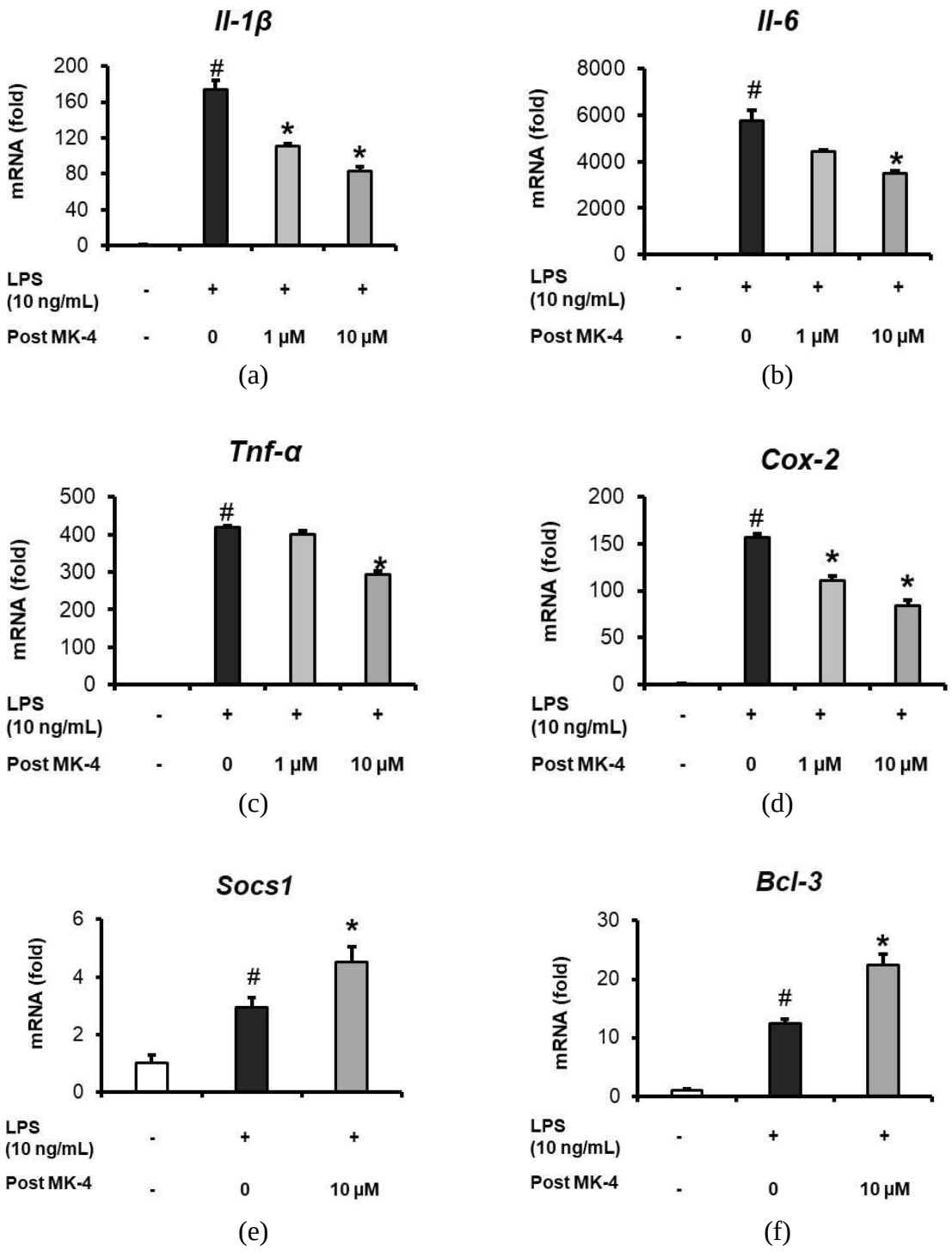
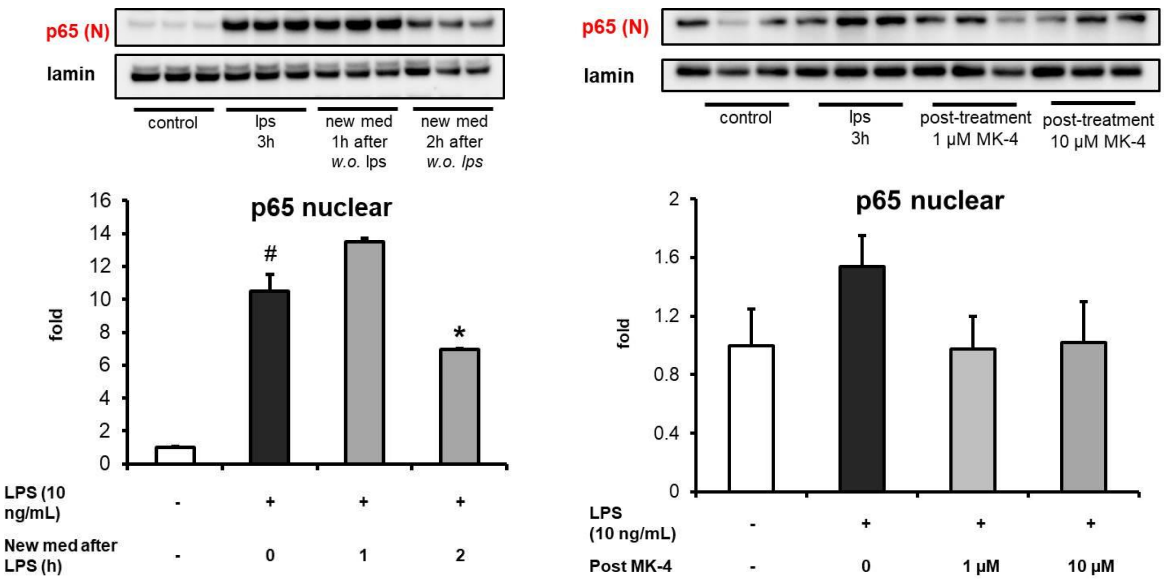


Fig. 8. Post-administration of MK-4 reduced the inflammatory mRNA expressions which were initiated by LPS and induced the negative regulator expressions. MG6 cells were first stimulated by LPS (10 ng/mL) for 3 h, then directly treated using different concentrations of MK-4 for 2 h. The mRNAs were analyzed by qRT-PCR, normalized to the levels of *Eef1a1* (elongation factor), and expressed as a fold of the control group. Data are presented as the mean ± S.E.M, $n = 3$; # $p < 0.05$ vs. untreated control; * $p < 0.05$ vs. LPS-only treated group.



(a) (b)

Fig. 9. Post-administration of MK-4 tended to reduce the nuclear level of NF-κB p65. MG6 cells were first stimulated by LPS (10 ng/mL) for 3 h, followed by changing of new fresh medium for different times incubation (a) or directly treated using different concentrations of MK-4 for 2 h (b). Data are presented as mean ± SE (n = 3), normalized to the lamin levels, and expressed as a fold of the control group. # $p < 0.05$ vs. untreated control; * $p < 0.05$ vs. LPS-only treated group.

Chapter 3

GGOH administration modulated the LPS-induced inflammatory reaction in MG6 mouse microglial-derived cells.

3.1. Introduction

Isoprenoid or terpenoid is a natural organic chemical consisting of five carbon compounds, isoprene, forming the plant's largest secondary metabolite. Many types of isoprenoids, such as geranylgeraniol (GGOH), farnesol (FOH), and phytol (POH), have been reported for their bioactive activities that attract pharmacological application (Kurtys et al., 2018; Silva et al., 2014). Moreover, isoprenoid is also a secondary metabolite in the mevalonate pathway. Therefore, the disruption of the isoprenoid synthesis due to the mevalonate kinase deficiency (MKD) condition has been associated with the inflammatory progression (Marcuzzi et al., 2010).

GGOH is a natural isoprenoid that shares a similar chemical structure with the side chain of MK-4. It is also contained in edible grains and vegetable oils. It was estimated that a Japanese person might consume approximately 500 μg/day of geranylgeranyl pyrophosphate (GGPP) from a staple food (rice) (Muraguchi et al., 2011). GGOH has been reported for its anti-inflammatory effect in the peripheral inflammatory models (Giriwono et al., 2019, 2013). Moreover, GGOH supplementation improved macrophage endotoxic tolerance (Kim et al., 2013). In the neuroinflammatory models, several studies reported the contribution of GGOH and geranylgeranylation on the protection of neuron and microglial cells from the side effect of statin treatments (Bi et al., 2004; Marcuzzi et al., 2016).

Based on the background mentioned above, we evaluated the effect of GGOH pre-treatment on the LPS-induced inflammation in MG6 mouse microglia derived cells. We aimed to investigate the anti-inflammatory effect of GGOH in a neuroinflammatory model independent from its mevalonate pathway. Here, we also focused on the NF-κB activity amelioration. The investigation on the anti-inflammatory effect of other isoprenoid analogs, including FOH and POH, was also performed. In addition, we attempted to analyze the GGOH treatment on the microglial phenotype polarization by measuring the M1 and M2 mRNA markers expressions.

3.2. Materials and Methods

3.2.1. Materials

GGOH was purchased from Sigma-Aldrich, while FOH and POH were provided by Wako Pure Chemicals. All the substances were dissolved in 99% ethanol at 100 mM concentration and stocked at -20ºC until used. The substance concentrations were adjusted to 0.1 % (v/v) for the experimental analysis. LPS (*Escherichia coli* O111: B4) was purchased from Sigma-Aldrich.

3.2.2. Cell Culture

As shown in chapter 2, section 2.2.2.

3.2.3. Cell Viability Evaluation

The cell viabilities were assessed using WST-1 or WST-8 method. Briefly, MG6 cells were seeded in a 96-wells plate and administered using GGOH in a different range of concentrations as indicated in the figure for 24 h. After the treatment, WST-1 (Takara Bio Inc., Shiga, Japan) or WST-8 premix was added, and the absorbances at 450 nm with a reference wavelength of 630 nm were measured using a spectrophotometer.

3.2.4. Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) Analysis

As shown in chapter 2, section 2.2.7.

3.2.5. Whole Cell Protein Extraction and Western Blot Analysis

As shown in chapter 2, section 2.2.6.

3.2.6. Fluorescence Microscopy

As shown in chapter 2, section 2.2.4.

3.2.7. Cytoplasmic-Nuclear Fractionation

As shown in chapter 2, section 2.2.5

3.2.8. Statistical Analysis

As shown in chapter 2, section 2.2.8. The Dunnett and Tukey-Kramer tests were used for multiple comparison analyses. The student's *t*-test was used for comparing two independent groups.

3.3. Results

3.3.1. GGOH inhibited the up-regulation of pro-inflammatory mRNA expressions

We first tested whether GGOH alone affected the MG6 cell viabilities. Using WST-1 assay, we found that GGOH up to 10 μM did not induce any toxicity in MG6 cells. However, the higher concentration (100 μM) clearly induced cytotoxicity in MG6 cells (Fig. 10). Hence, in the following evaluation, we set the GGOH concentration up to 10 μM. Thereafter, we evaluated the GGOH pre-treatment on the pro-inflammatory cytokine mRNA expressions. We observed that GGOH dose-dependently down-regulated the mRNA expressions of *Il-1β*, *Il-6*, *Tnf-α*, and *Cox-2* compared to the LPS group without GGOH supplementation, as shown in Fig. 11a-d. Moreover, we also analyzed the GGOH effect in different preincubation times. As shown in Fig. 12a-c, the incubation of GGOH for 24 h demonstrated the optimum effect. In comparison, its pre-administration under 24 h of incubation showed different effects on the inhibition against LPS-induced pro-inflammatory cytokine expressions. We confirmed these results by comparing the NF-κB-related protein expressions in incubating 0 h and 24 h. Fig. 12d-e showed that the phosphorylation of NF-κB p65 was inhibited and IκBα level was maintained in 24 h incubation than 0 h of GGOH incubation. Therefore, we used 24 h for GGOH incubation in the subsequent analysis.

3.3.2. GGOH mitigated the NF-κB activity in MG6 cells

We analyzed the molecular mechanism underlying GGOH anti-inflammatory action by profiling the NF-κB-related protein expressions. The GGOH pre-treatment, especially at 10 μM, effectively modulated the phosphorylation of TAK1, IKKα/β, and p65 with regard to their total protein levels, compared to the LPS administration alone. In addition, the evaluation of the GGOH effect on the inhibitor of NF-κB (IκBα) was also performed. LPS administration was found to induce IκBα phosphorylation significantly and provoked its degradation. On the other hand, the GGOH pre-treatment effectively blocked the phosphorylation of IκBα while maintaining its cellular level (Fig. 13). Altogether, these results indicated the function of GGOH on mitigating NF-κB activation in LPS-induced MG6 cells.

3.3.3. GGOH inhibited the NF-κB p65 nuclear translocation

As we mentioned in the introduction and chapter 2, the NF-κB activation is associated with the nuclear translocation of its p65 subunit. Here, we also checked the GGOH effect on

this molecular signaling. In fluorescence microscopy analysis, we again found that NF-κB p65 was mainly detected in the cytoplasmic part under normal situations. LPS administration induced solid p65 staining in the nuclear part, suggesting the nuclear transfer process. On the other hand, GGOH pre-treatment markedly reduced the p65 staining in the nuclear part, indicating its inhibitory effect against NF-κB nuclear localization (Fig. 14a). We also observed a similar result in confirmation analysis using western blot for estimating cytoplasmic and nuclear NF-κB p65 levels. We found significant levels of p65 in a nuclear fraction under the administration of LPS alone, while GGOH effectually reduced this parameter. In cytoplasmic counterpart, we did not find any difference of NF-κB p65 among the group treatments, as shown in Fig. 14b-c.

3.3.4. GGOH modulated the NF-κB upstream regulators

LPS mediates the NF-κB signaling through the recruitment of the upstream regulators. After activation of TLR4, the MYD88 was recruited together with the IRAK1. This recruitment allows the phosphorylation of TRAF6, which conveys signal through TAK1-TAB1/2 complex to induce IKKα/β phosphorylation and activates NF-κB (Li and Verma, 2002). This experiment detected that LPS administration reduced the IRAK1 and TRAF6 expressions. On the contrary, the GGOH pre-treatment retained the IRAK1 expression but not the TRAF6 expression (Fig. 15a). In Fig. 15b, we further analyzed the *Irak1* and *Traf6* mRNA expressions. However, we did not indicate any significant difference between LPS administration alone and GGOH pre-treatment plus LPS. It suggested that the involvement of GGOH on the upstream regulators was independent of transcriptional activity. In addition, the GGOH treatment alone was found to cause degradation on IRAK1 and TRAF6 proteins in THP-1 cells. Here, we administered GGOH without any LPS in MG6 cells, but we did not observe a similar degradation effect on IRAK1 and TRAF6 in all-time administrations (Fig. 16).

3.3.5. GGOH and other isoprenoid analogs ameliorated the LPS-induced pro-inflammatory cytokine expressions

The isoprenoid analogs have been known for their anti-inflammatory activities (Kurtys et al., 2018). In a previous experiment using LPS-induced THP-1 human macrophage-like cells, only GGOH but other isoprenoids were found to inhibit the inflammation (Ohsaki et al., 2010). In this study, we utilized GGOH and two other isoprenoids, FOH and POH, to modulate the LPS-induced inflammation in MG6 cells. Fig.

17a-d demonstrated that all isoprenoid analogs effectively prevented the up-regulation of pro-inflammatory cytokine mRNA expressions. Only did POH not mitigate the *Il-1β* mRNA expression. These results implied that other isoprenoid analogs might also have anti-inflammatory potentials. The difference in the isoprenoid effect between the current and previous studies might be due to the cell line used. Nevertheless, our results supported the previous hypothesis considering the pharmacological function of isoprenoids.

3.3.6. GGOH effect on the M2 phenotype markers in MG6 cells

As already mentioned, microglia can be transformed into distinct phenotypes by specific stimuli. Under anti-inflammatory stimulation, microglia would be polarized to the M2 phenotype, which expresses anti-inflammatory and wound healing markers. Here, we attempted to evaluate the effect of GGOH administration on the M1/M2 polarization by measuring the M1 and M2 mRNA markers. Fig. 18 showed that LPS administration directed the *iNOS* mRNA expression as an M1 marker, while GGOH pre-treatment inhibited this up-regulation. On the other hand, we detected that LPS administration reduced *Arg-1* mRNA expression as the M2 marker, while GGOH pre-treatment significantly up-regulated this expression. Importantly, GGOH treatment alone effectively induced *Arg-1* and *Ym-1* mRNA expressions as M2 markers for a total of 27 h of incubation (Fig. 19a-b). These results suggested the hindering effect of GGOH treatment on the M1 marker and the inducing effect on the M2 markers. In addition, compared to the non-treated group, GGOH induced the inflammatory negative regulator's expressions, including *Socs1* and *Bcl-3*, as shown in Fig. 19c-d.

3.4. Discussion

This study demonstrated the beneficial effect of GGOH pre-treatment on the inhibition against the LPS-induced MG6 cells, focusing on the NF-κB signaling pathway. The other isoprenoid analogs, including FOH and POH, showed an anti-inflammatory effect. Moreover, we observed a marked regulation of GGOH administration on the polarization of MG6 cells into the M2 phenotype.

In previous studies, GGOH administration was found to suppress inflammatory markers in rat plasma and medulloblastoma cells (Giriwono et al., 2013; Marcuzzi et al., 2016). Here we also observed down-regulation effects on the expressions of *Il-1β*, *Il-6*, *Tnf-α*, and *Cox-2*, supporting that GGOH promotes anti-inflammatory action in peripheral and

nervous system models. Moreover, the incubation of GGOH under 24 h showed a more negligible effect. The possibility behind this feature is that during the short incubation, the production of the anti-inflammatory substances might still not be fully induced by GGOH. Another possibility is that sufficient incubation times are required for GGOH conversion into its metabolite, which might have more anti-inflammatory properties (Giriwono et al., 2019). In the second case, a statin treatment that suppresses the endogenous isoprenoid production followed by administration of GGOH is thought to be helpful to clarify the GGOH conversion.

We demonstrated here that GGOH pre-treatment inhibited the NF-κB signaling pathway through the suppression of TAK1, IKKα/β, p65, and IκBα phosphorylation. Furthermore, it maintained the total IκBα level and prevented the NF-κB p65 nuclear translocation. However, although there was clear evidence of NF-κB inhibition, we still could not categorize the GGOH target molecule in this pathway. Therefore, molecular docking considering the interaction of GGOH and NF-κB proteins might be beneficial. In another perspective, some isoprenoid analogs were reported to target the IKKα/β protein. For example, Artemisolide and Triterpenoid CDDO-Me were found to interact and trigger the oxidation of cysteine-179 residue of IKKβ, respectively (Ahmad et al., 2006; Kim et al., 2007). In the current study, we also noticed the reduction of IKKβ expression in GGOH treatment. Therefore, there is also a possibility that GGOH target the Cys-179 residue. Hence, the further experiment using GGOH and recombinant IKKβ by replacing Cys-179 with Ala residue is worth considering.

In the upstream regulators, LPS administration reduced IRAK1 and TRAF6 expressions, while GGOH retained them. It was likely that inflammatory reaction triggered a negative feedback loop by degrading the signal regulators to limit the further inflammatory effect (Gottipati et al., 2008; Yamin and Miller, 1997). Since the GGOH already suppressed the inflammation, negative feedback might not occur, and the regulator levels were preserved. Another plausible explanation is that LPS might modify the IRAK1 and TRAF6 interaction. Previous studies reported that LPS led the K63-linked polyubiquitination of IRAK1, which mediated its binding to NEMO, the regulatory subunit of IKK families. This conformation was thought to cause poor detection of unmodified IRAK1 under LPS influence in immunoblot analysis (Conze et al., 2008; Windheim et al., 2008). In GGOH administration, the IRAK1 expression was retained because GGOH might interfere with the LPS effect on the IRAK1 and NEMO interaction.

The administration of isoprenoid analogs, including GGOH, FOH, and POH, effectively inhibited the pro-inflammatory cytokine expressions in LPS-induced MG6 cells. The slight difference where POH did not reduce the *Il-1β* mRNA expression was still unclear. However, previous studies considered that the isoprenoids functions were affected by several factors such as cell penetration ability, protein prenylation, enzymatic reactions, and further molecular interaction (Marcuzzi et al., 2010; Miquel et al., 1998). This study supposed that all used isoprenoid analogs could infiltrate the MG6 cells. However, there were possibilities that the target molecules of GGOH, FOH, and POH were differents. For the comparison, another study reported that γ-tocotrienol, a member of vitamin E with the unsaturated chains, but not γ-tocopherol, could block the NF-κB activity (Ahn et al., 2007). Nonetheless, our study supported the proposal of isoprenoids as natural NF-κB inhibitors (Salminen et al., 2008).

We found that single GGOH administration inhibited the up-regulation of the M1 marker, induced the M2 mRNA markers for a total of 27 h of time administration. It hints that the time incubation might be an important factor to consider the GGOH effect on the microglial polarization. Moreover, the concept of M1/M2 microglia was still questioned and considered the over-simplification. The actual microglial heterogeneities in the CNS are immensely complex (Subhramanyam et al., 2019; Tang and Le, 2016). Therefore, further examination using in vivo experiment is necessary in this regard.

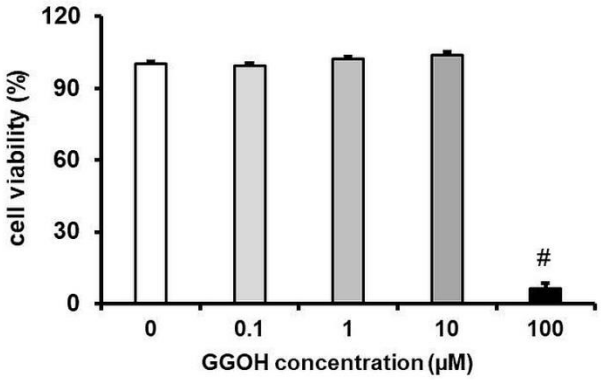


Fig. 10. The administration of GGOH did not affect the MG6 cells viabilities. MG6 cells were administered with GGOH at different concentrations for 24 h. Cell viabilities were then analyzed using the WST -1 method. Data are presented as the mean ± S.E.M, $n = 3$; # $p < 0.05$ vs. untreated control. (cited from Saputra et al., Int. J. Mol. Sci. 2021, 22, 10543).

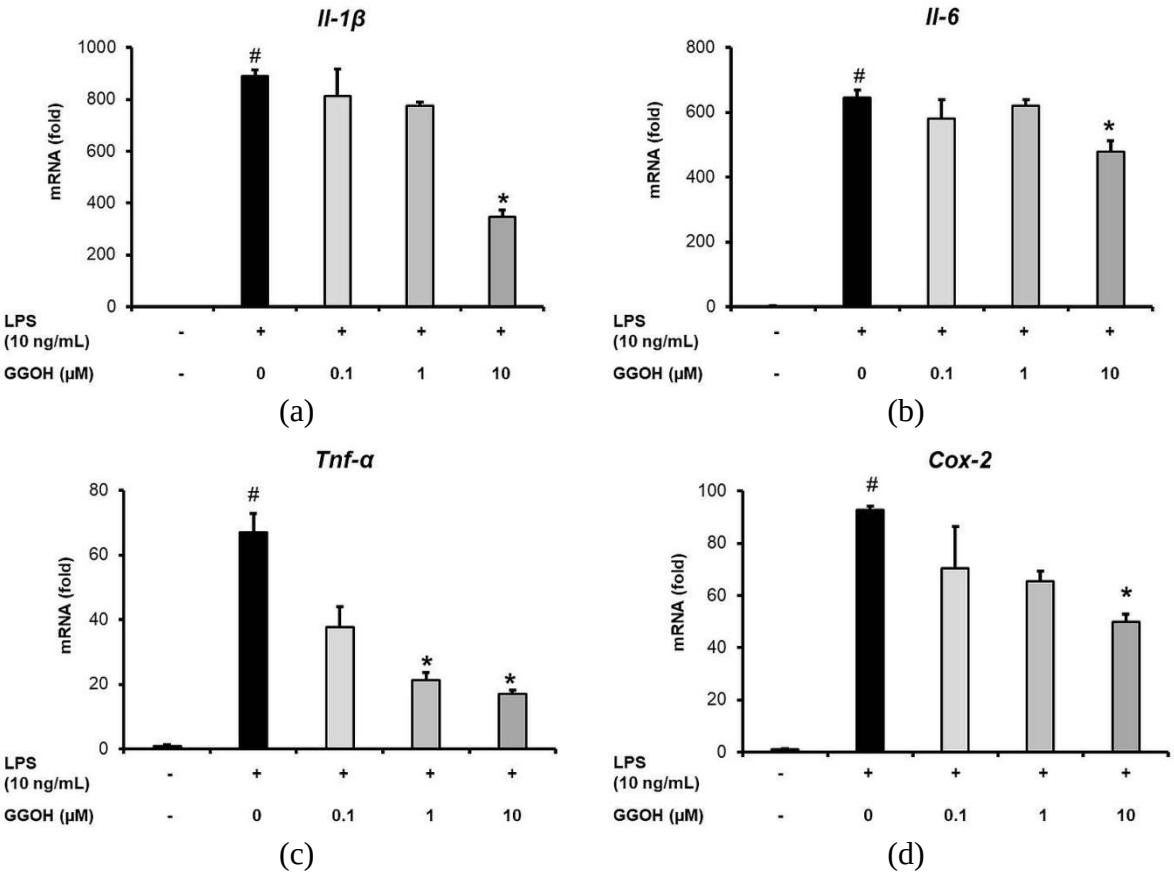


Fig. 11. The down-regulation of pro-inflammatory cytokine mRNA expression by GGOH. MG6 cells pre-treated with GGOH at different concentrations for 24 h followed by LPS (10 ng/mL) treatment for 3 h. The pro-inflammatory cytokines mRNA levels were analyzed via qRT-PCR, normalized to *Eef1a1* (elongation factor), and expressed as fold-changes relative to control group. Data are presented as the mean ± S.E.M, $n = 3$; # $p < 0.05$ vs. untreated control; * $p < 0.05$ vs. LPS-only treated group). (cited from Saputra et al., Int. J. Mol. Sci. 2021, 22, 10543).

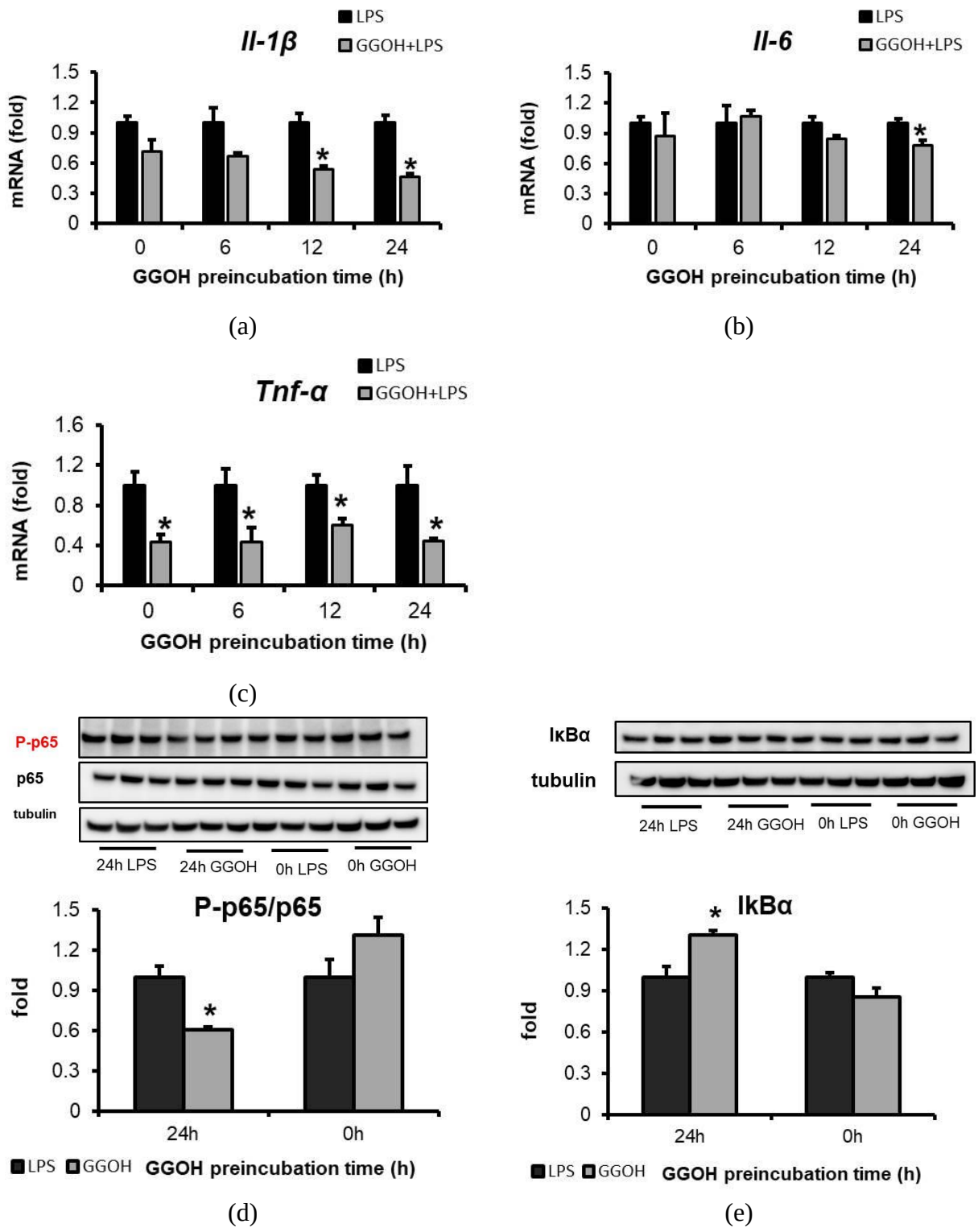


Fig. 12. Pro-inflammatory cytokine mRNA and NF-κB expression levels in different GGOH preincubation times. MG6 cells pre-treated with GGOH (10 μM) at different times followed by LPS treatment (10 ng/mL) for 3 h. Black bar, LPS-only treated groups; grey bar, GGOH pre-treated groups. The mRNA levels were measured via qRT-PCR, normalized to *Eef1a1* (elongation factor), while protein was analyzed using western blot and normalized to α-tubulin. The values are expressed as fold-changes relative to LPS-only treated MG6 cells. Data are presented as the mean ± S.E.M, $n = 3$; # $p < 0.05$ vs. untreated control; * $p < 0.05$ vs. LPS-only treated group. (cited from Saputra et al., Int. J. Mol. Sci. 2021, 22, 10543).

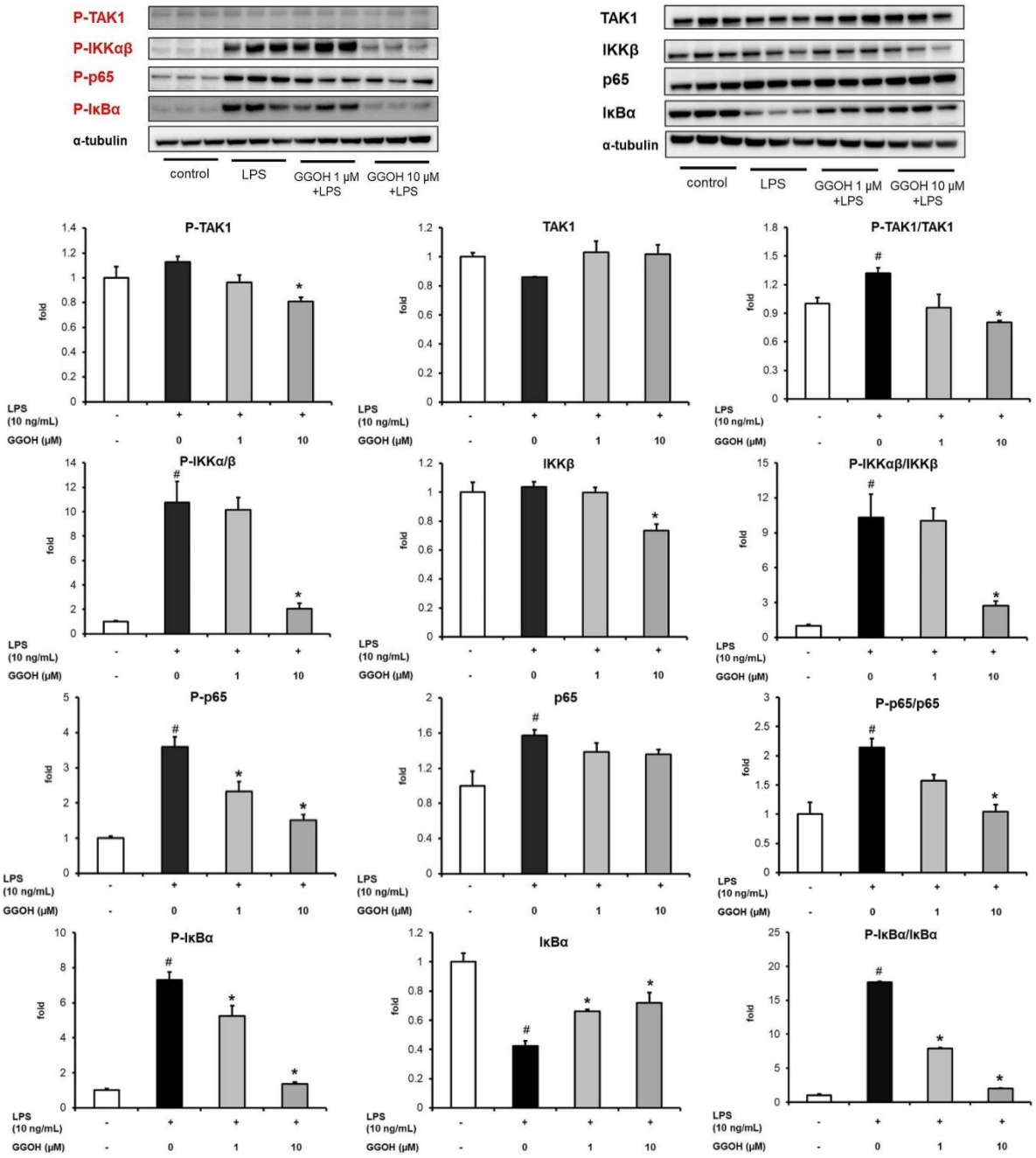
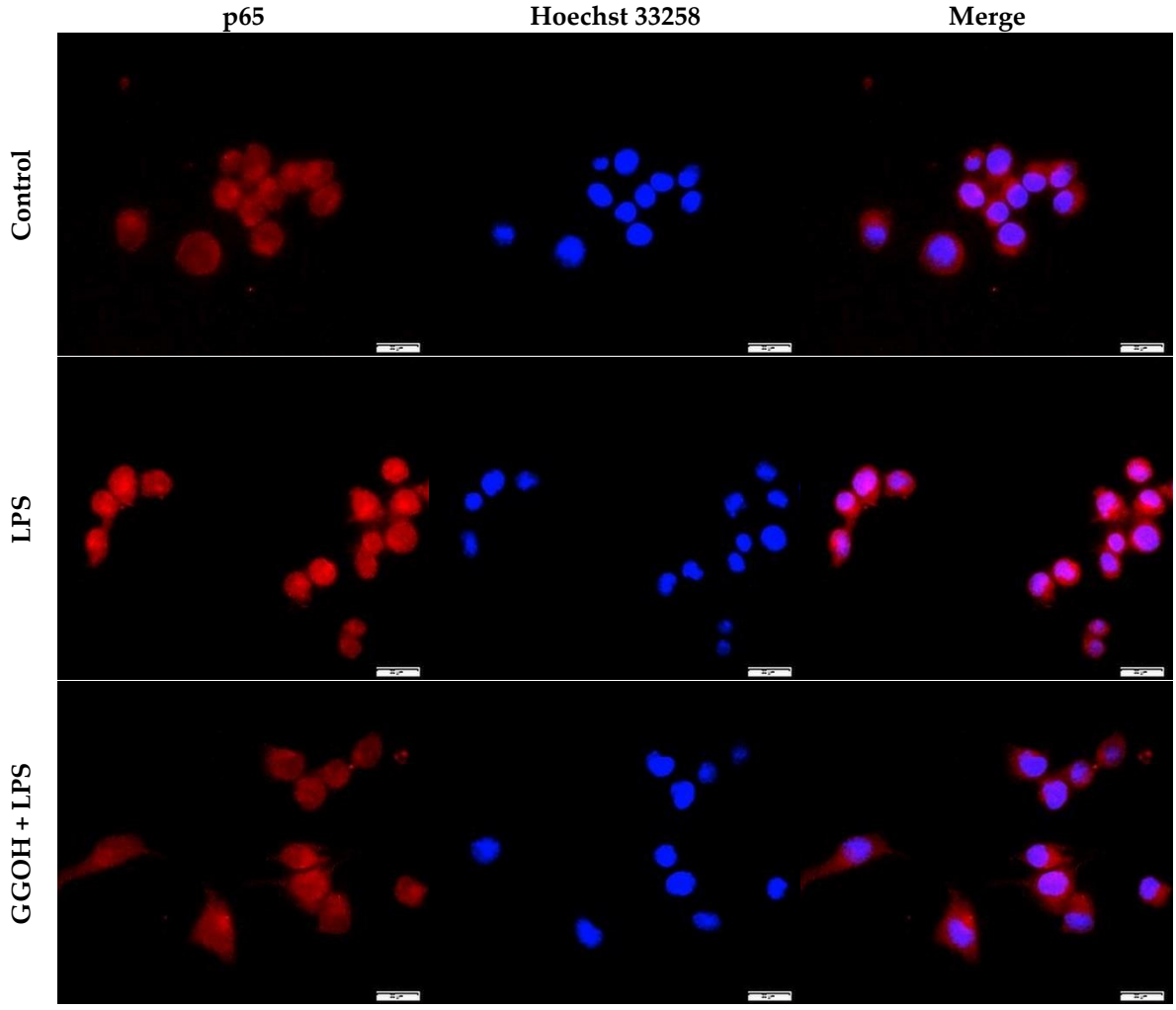
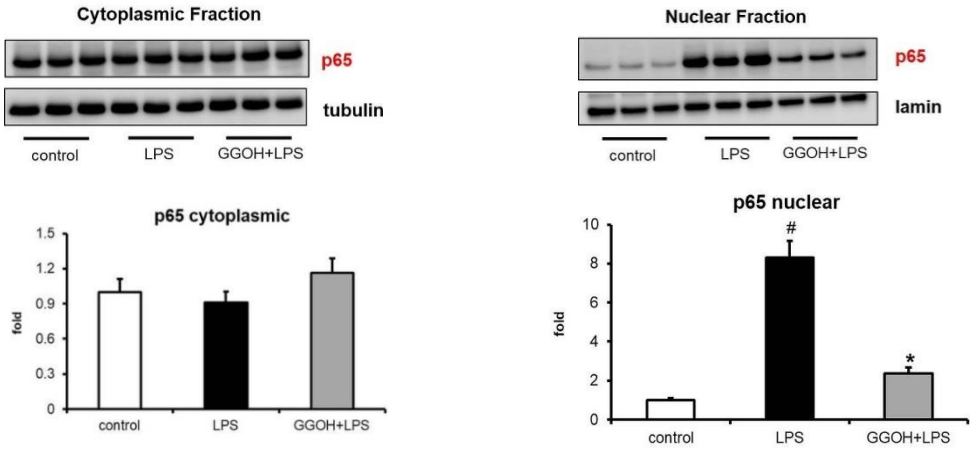


Fig. 13. The down-regulation of NF-κB-related proteins expressions by GGOH administration. MG6 cells were pre-treated with GGOH for 24 h followed by LPS treatment (10 ng/mL) for 1 h. Western blot was used to evaluate the expressions of TAK1, IKKα/β, p65, and IκBα. Data are presented as the mean ± SEM, $n = 3$, normalized to the total protein levels detected based on α-tubulin. # $p < 0.05$ vs. untreated control; * $p < 0.05$ vs. LPS-only treated group. (cited from Saputra et al., Int. J. Mol. Sci. 2021, 22, 10543).

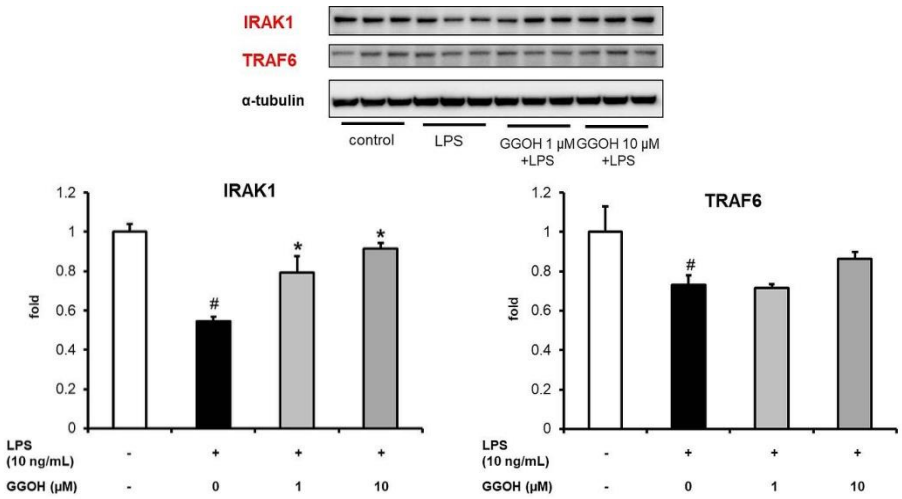


(a)



(b) (c)

Fig. 14. The blocking of NF-κB p65 nuclear translocation by GGOH administration. MG6 cells were pre-treated with GGOH (10 μM) for 24 h followed by LPS treatment (10 ng/mL) for 1 h. (a) Cells were stained with anti-p65 (red) antibodies. Nuclei were stained with Hoechst 33258 (1 μg/mL) (blue). Scale bars, 20 μm; (b) and (c) The cytoplasmic and nuclear fractions of treated MG6 cells analyzed by western blot analysis. Data are presented as the mean ± SEM, $n = 3$, normalized to total protein levels based on the use of α-tubulin or lamin, and expressed as the fold-change relative to the values corresponding to the control cells. # $p < 0.05$ vs. untreated control. * $p < 0.05$ vs. LPS-only treated group. (cited from Saputra et al., Int. J. Mol. Sci. 2021, 22, 10543).



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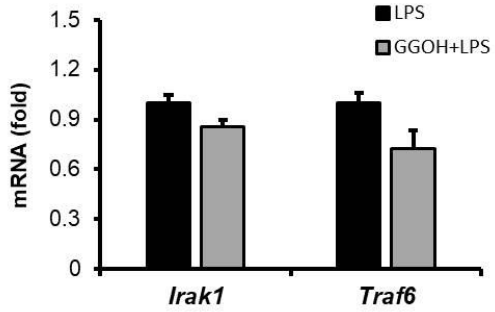


(b)

Fig. 15. The involvement of GGOH in the upstream regulators of NF-κB signaling. MG6 cells were pre-treated with GGOH for 24 h followed by LPS treatment (10 ng/mL) for 1 h. (a) Western blot images and quantifications of IRAK1 and TRAF6 expression levels in the presence of GGOH followed by LPS treatment for 1 h. (b) *Irak1* and *Traf6* mRNA expression levels after 1 h of LPS treatment. Total cell lysates were collected and subjected to western blot analysis or qRT-PCR. Data are presented as the mean ± SEM, $n = 3$, normalized to total protein levels detected based on α-tubulin or *Eef1a1*. # $p < 0.05$ vs. untreated control; * $p < 0.05$ vs. LPS-only treatment. (cited from Saputra et al., Int. J. Mol. Sci. 2021, 22, 10543).

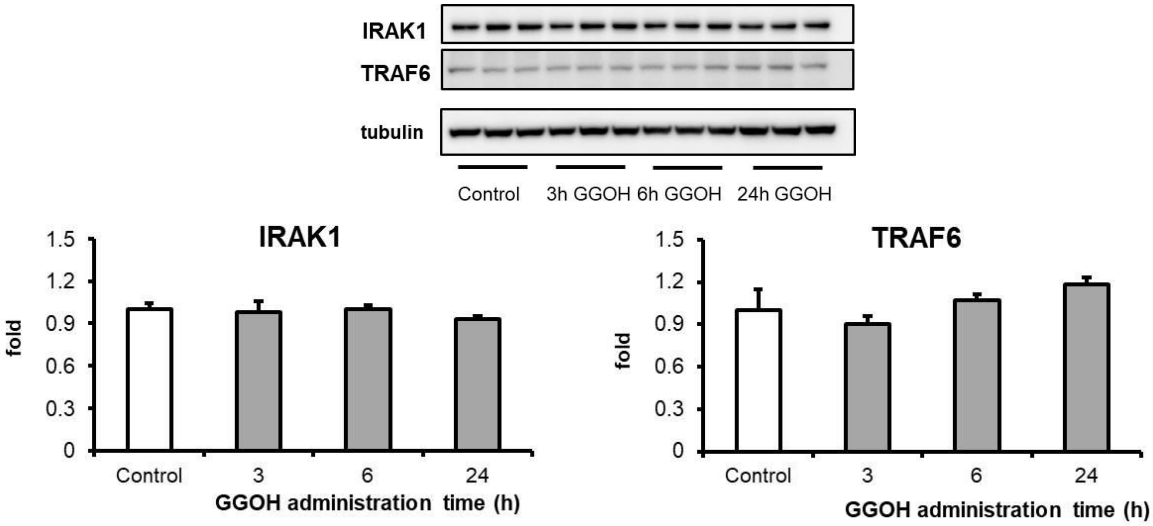


Fig. 16. Geranylgeraniol (GGOH) did not affect the IRAK1 and TRAF6 expressions in non LPS condition. Cells were treated with GGOH for different times and the protein expressions were analyzed with western blot. Data are presented as the mean ± SEM, $n = 3$, normalized to total protein levels detected by α-tubulin and expressed as the fold-change relative to control cell values. (cited from Saputra et al., Int. J. Mol. Sci. 2021, 22, 10543).

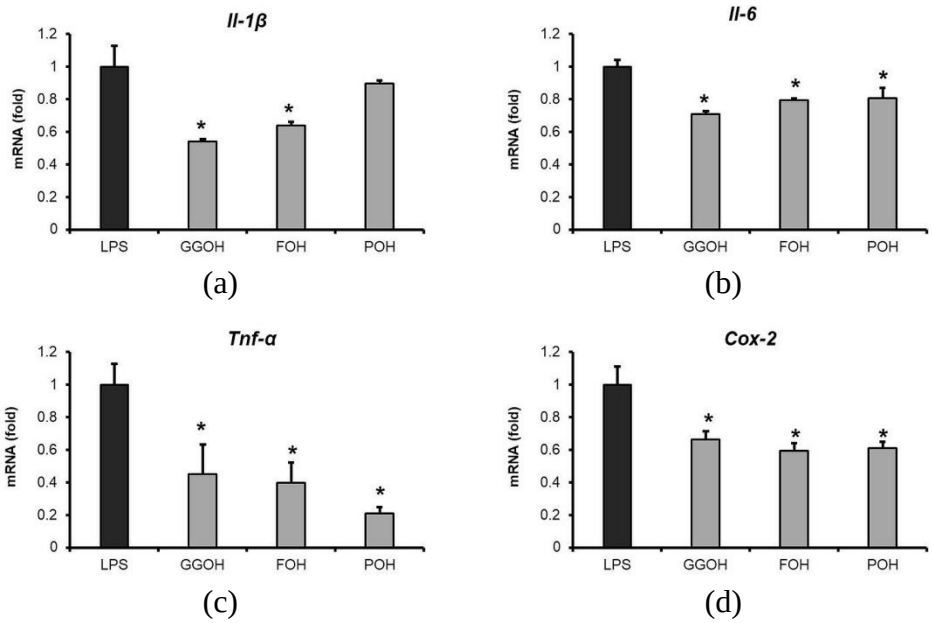


Fig. 17. The down-regulation of pro-inflammatory cytokines mRNA by isoprenoid analogs. MG6 cells were administered isoprenoid analogs (10 µM) for 24 h followed by LPS treatment (10 ng/mL) for 3 h. mRNA levels were analyzed via qRT-PCR, normalized to the level of *Eef1a1* (elongation factor), and expressed as fold-changes relative to the LPS-only treated group. Data are presented as the mean ± S.E.M, $n = 3$; * $p < 0.05$ vs. LPS-only treatment group. GGOH, geranylgeraniol; FOH, farnesol; and POH, phytol. (cited from Saputra et al., Int. J. Mol. Sci. 2021, 22, 10543).

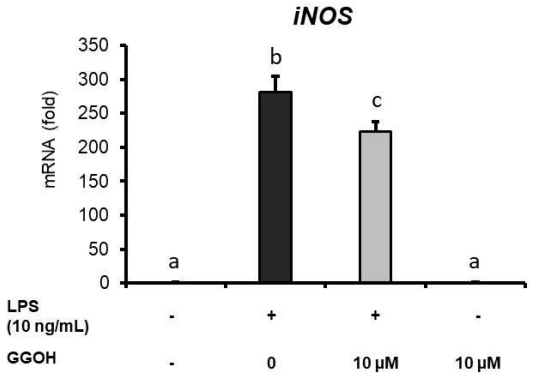


Fig. 18. The inhibition of the M1 marker up-regulation induced by LPS by GGOH administration. MG6 cells were pre-treated with or without GGOH (10 μM) for 24 h followed by LPS (10 ng/mL) for 3 h. mRNA expression levels were analyzed via qRT-PCR, normalized to the level of *Eef1a1* (elongation factor), and expressed as the fold-change relative to the values corresponding to the control group. Data are presented as the mean ± S.E.M, $n = 3$; $p < 0.05$ vs. control; different letters indicated the significant differences.

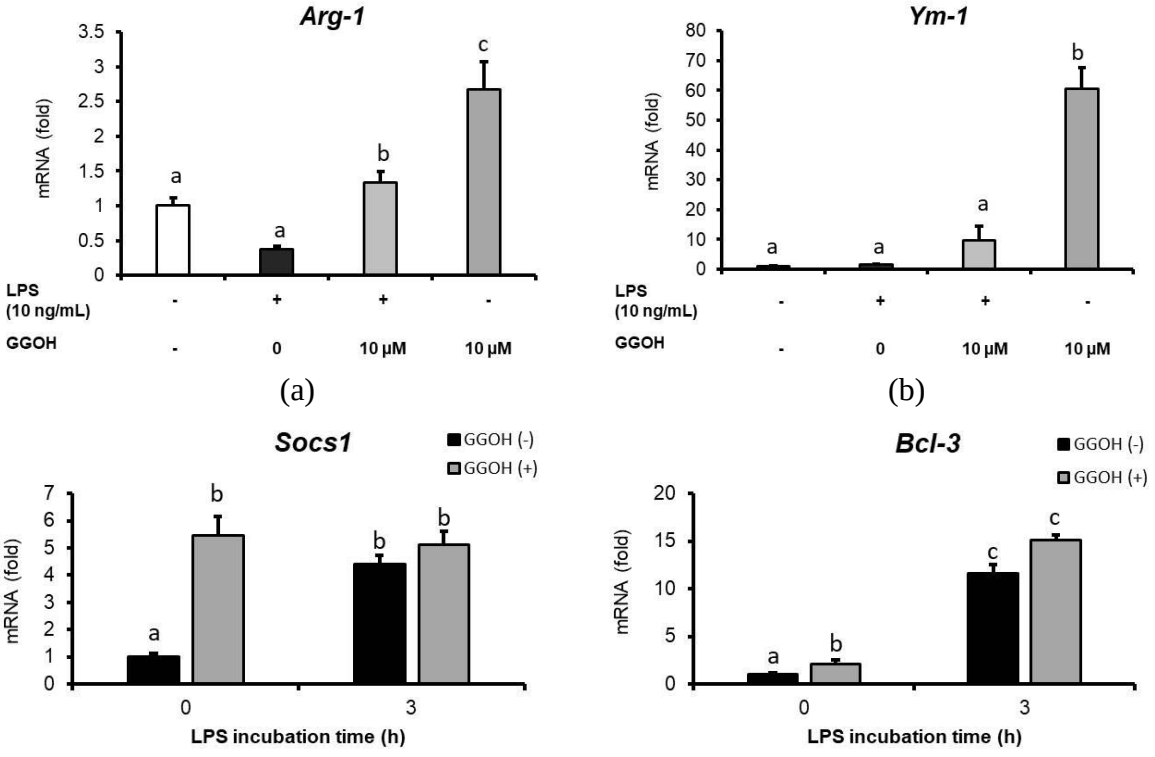


Fig. 19. The induction of the M2 markers and negative regulators mRNA expressions by GGOH administration. MG6 cells were pre-treated with or without GGOH (10 μM) for 24 h followed by LPS (10 ng/mL) for 0 h or 3 h. mRNA expression levels were analyzed via qRT-PCR, normalized to the level of *Eef1a1* (elongation factor), and expressed as the fold-change relative to the values corresponding to the control group or LPS treated without GGOH. Data are presented as the mean ± S.E.M, $n = 3$; $p < 0.05$ vs. control; different letters indicated the significant differences.

Chapter 4

GGOH prevented the microglial-mediated neurotoxicity in HT22 hippocampal neuron cells

4.1. Introduction

The ultimate consequence of neuroinflammation is the neurodegenerative process that contributes to dementia. During neurodegeneration, the central nervous system undergoes progressive neuron cell loss via the cell death mechanisms such as necrosis and apoptosis (Guadagno et al., 2013). Since microglia are the key actor in neuroinflammation, they were suggested to play a role in excessive neuron cell death directly. There are several hypotheses considering the mechanisms of neuron cell death induced by microglia, including production of superoxide and derivative oxidants, synthesis of inducible nitric oxide synthase (iNOS) and NO production, production of glutamate, glutaminase, TNF-α, and cathepsin B, phagocytosis of neuron and less production of neurotrophic factors (Brown and Vilalta, 2015).

The factors produced by microglia during neuroinflammation might induce neuron cells death directly or indirectly. For example, TNF-α and IL-1β produced by LPS-activated microglia have been reported to induce apoptosis and damaged cell regeneration in neural precursor cells (Guadagno et al., 2015, 2013). Another study reported the synthesis effect of IL-6 complex on the amyloid β precursor protein, a hallmark of Alzheimer's disease (Ringheim et al., 1998). Moreover, NO production was also associated with apoptosis in neuron cells through the endoplasmic reticulum stress induction and cell energy disruption (Borutaite et al., 2000). In addition, elevated inflammatory mediators were also found in the cerebrospinal fluid of neurodegenerative patients (Khaibullin et al., 2017).

This study aimed to investigate the further effect of GGOH pre-treatment on the inhibition against microglial-mediated neurotoxicity in HT22 mice hippocampal neuronal cells. The previous study has shown the beneficial effect of MK-4 treatment to protect the SHSY5Y neuroblastoma cells from the detrimental effect of conditioned medium from LPS-induced BV2 microglial cells (Yu et al., 2016). In this study, we also utilized conditioned medium analysis, assuming that the inflammatory mediators produced by LPS-induced MG6 cells were contained in cell media and could induce toxicity in neuron cells. In addition, we used indirect interaction scenarios between activated MG6 cells and healthy MG6 cells and activated MG6 cells and healthy HT22 cells.

4.2. Materials and Methods

4.2.1. Materials

As described in chapter 3, section 3.2.1

4.2.1. Cell Cultures

MG6 cells were used as described in chapter 2, section 2.2.2. In addition, mouse hippocampal HT22 neuron cells were obtained from Sigma-Aldrich. The HT22 cells were cultured in a DMEM medium (Sigma) containing 10% fetal bovine serum (Biowest, Nualliè, France) and penicillin-streptomycin solution. The cells were maintained in humidified atmosphere incubator with 5% CO2 at 37 ºC and passaged when reaching approximately 80% confluency.

4.2.2. Conditioned Medium Experiments

For conditioned medium (CM) experiments, MG6 cells were treated with or without GGOH followed by LPS administration for the indicated times. The media then were collected to incubate another batch of MG6 cells or HT22 cells, as mentioned in the figure captions. After finished the CM incubation, the cells were subject to further analysis.

4.2.3. Cell Viability Evaluation

As described in chapter 3, section 3.2.3.

4.2.4. Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) Analysis

As shown in chapter 2, section 2.2.7.

4.2.5. Whole Cell Protein Extraction and Western Blot Analysis

As shown in chapter 2, section 2.2.6.

4.2.6. Propidium Iodide Staining

HT22 cells were incubated with the CM from MG6 cells. After the indicated times, the medium was removed, washed several times with PBS, and incubated using propidium iodide (PI, Dojindo, Kumamoto, Japan) in PBS for 15 minutes without light exposure. Cells were then visualized using a fluorescence microscope system (Olympus IX81, Japan).

4.2.7. Statistical Analysis

As shown in chapter 3, section 2.2.8.

4.3. Results

4.3.1. GGOH inhibited conditioned medium-induced inflammation in MG6 cells

We initially investigated whether LPS conditioned medium (LPS_CM) can enhance inflammation in healthy MG6 cells. After LPS administration in MG6 cells, we collected the cell media as the CM and used it to incubate another batch of normal MG6 cells for 3 h. Fig. 20a-c revealed that LPS_CM significantly up-regulated the mRNA expressions of *Il-1β*, *Il-6*, and *Tnf-α* in the new batch of MG6 cells compared to the direct LPS administration. Moreover, the pre-treatment of GGOH on the first batch of MG6 cells clearly modulated the enhancing pro-inflammatory cytokine mRNA expressions in the LPS_CM-induced new batch of MG6 cells. These results indicated that GGOH pre-treatment reduced the pro-inflammatory factor production induced by LPS which triggered further inflammation in the healthy cells.

4.3.2. GGOH protected the HT22 cells from the LPS-induced MG6 mediated neurotoxicity

Given that the microglial-derived inflammatory factors can induce neurotoxicity, we then applied the CM experiment using HT22 mouse hippocampal neuron cells (Fig. 21a). We first evaluated the GGOH effect on the HT22 cell viabilities. Fig. 21b showed that GGOH until 10 μM did not induce toxicity in HT22 cells, indicating that GGOH itself was not a cell death initiator in these concentration ranges. Then, we compared the effect between direct LPS administration and LPS_CM from MG6 cells on HT22 cells viabilities by WST-1 method. As shown in Fig. 21c, the direct LPS administration slightly reduced HT22 cell viabilities. However, the LPS_CM from MG6 cells showed more detrimental effects by the lower HT22 cell viabilities, especially at 1 μg/mL of LPS concentration. In Fig 21d, we tried to pre-treat MG6 cells using GGOH before LPS induction and CM incubation in HT22 cells. We observed that without GGOH pre-treatment in MG6 cells, the viability of HT22 was the lowest. On the other hand, pre-treatment of GGOH in MG6 cells effectively maintains the HT22 cell viabilities suggesting that GGOH reduced the toxicity effect of MG6 cells LPS_CM.

We also analyzed the effect of LPS_CM using propidium iodide (PI) staining in HT22 cells. PI would infiltrate the membranes of death or dying cells, but it can not penetrate the live cells (Crowley et al., 2016). Under LPS_CM from MG6 cells, HT22 cells demonstrated the high intensity of PI fluorescence. However, when the GGOH was pre-treated in MG6 cells, the PI intensity in HT22 cells could be reduced, as shown in Fig. 21e. Moreover, the MG6 cells LPS_CM was found to induce phosphorylation in cell death markers, including ERK1/2 and p38, in HT22 cells. Conversely, GGOH pre-treatment in MG6 cells significantly inhibited the ERK1/2 and p38 phosphorylations in HT22 cells (Fig. 22). These data stipulated that GGOH prevented HT22 neuronal cell toxicity mediated by inflammatory factors from activated MG6 cells.

4.4. Discussion

This experiment observed that GGOH pre-treatment inhibited further inflammation in healthy MG6 cells incubated with medium from LPS-induced MG6 cells (LPS_CM). Moreover, GGOH also indirectly maintains the HT22 cell viabilities against the detrimental effect of LPS_CM. We believed that the LPS_CM contained LPS and the inflammatory cytokines produced by MG6 cells. These cytokines were responsible for inducing neurotoxicity. HT22 cells were reported to possess TLR4, IL-1 receptor, and TNF receptor, which might trigger the cytokines triggering a neurotoxic signaling cascade (Alboni et al., 2014; Guan et al., 2019; Olianas et al., 2019). Since GGOH had already reduced the pro-inflammatory cytokines in MG6 cells, the further adverse effect in HT22 cells could be inhibited. Another possibility was that GGOH or its metabolites were also contained in the conditioned medium and directly improved the signaling cascade in HT22 cells. In this hypothesis, the immediate treatment of GGOH on conditioned medium-induced HT22 cells is worth considering.

As mentioned in the above introduction, there are two types of neuron cell death conditions, necrosis, and apoptosis. In the necrosis event, the cell death is triggered by an unregulated process such as mechanistic injuries and pathogen infection. On the other hand, apoptosis forms programmed cell death through the tightly cellular responses (Fink and Cookson, 2005). Here we utilized PI staining, which serves as one alternative to detect the necrotic cells (Unal Cevik and Dalkara, 2003). Although we found that LPS_CM increased the PI staining and GGOH pre-treatment inhibited this event, we only detected fewer PI-positive cells in visual evaluation. However, we found significant results in the measurement

of apoptosis markers, ERK1/2 and p38, in western blot analysis. These results indicated that LPS_CM might be induce neurotoxicity in HT22 cells via the regulated pathway (apoptosis) than via the un-regulated process (necrosis). The evaluation using flow-cytometry for measuring both apoptosis and necoris markers in one cell batch is essential. Nevertheless, the GGOH pre-treatment in MG6 cells prevented the up-regulation of cell death markers in LPS_CM-induced HT22 cells.

In addition, GGOH itself was previously suggested to induce the apoptosis event (Takeda et al., 2001). However, the pro-apoptotic effect of GGOH was achieved in liver cancer cell lines. In another study, GGOH was even found to protect the myeloma cells from incadronate-induced apoptosis (Shipman et al., 1998). Here, in colorimetric analysis, we showed that up to 10 µM of concentration, GGOH did not induce toxicity in HT22 cells. However, the higher concentrations reduced the cell viability. Hence, we assumed that the pro-apoptotic properties of GGOH might be a cell-specific case. Another important point is that the GGOH treatment should consider a safe dose that does not affect cell viability. Fortunately, we demonstrated that GGOH in 10 µM dose has already denoted promising effects.

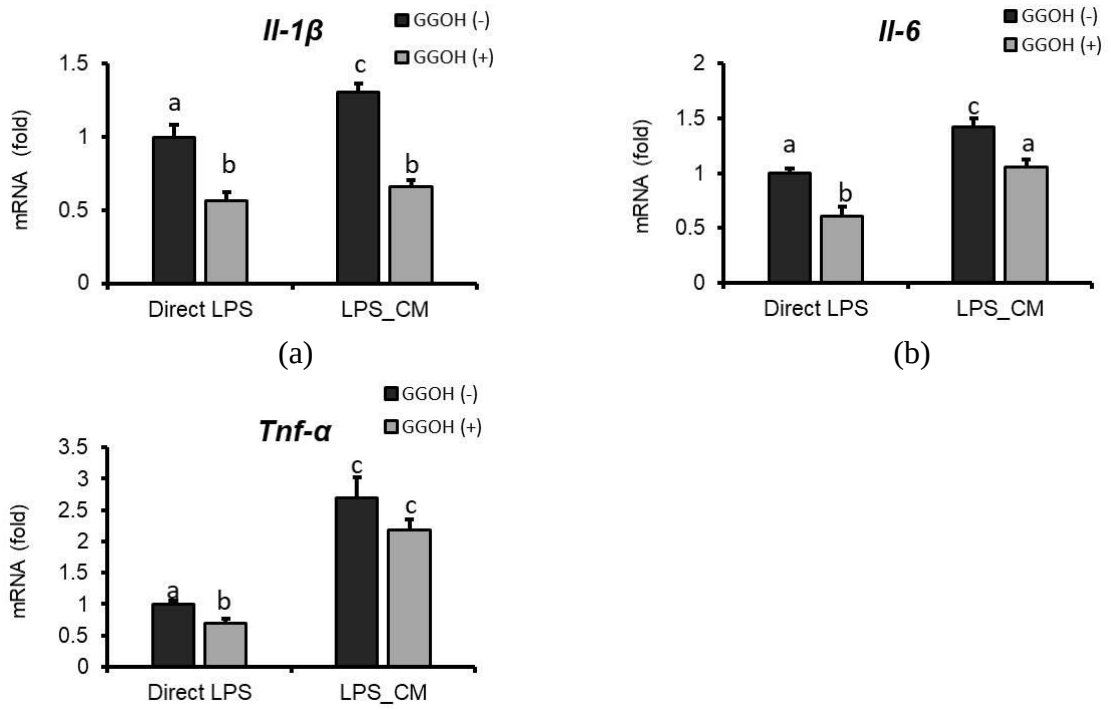


Fig. 20. Inhibition on the effect of LPS conditioned medium (LPS_CM) by GGOH on the new batch of MG6 cells. MG6 cells were incubated with GGOH (10 µM) for 24 h followed by LPS (10 ng/mL) for 3h as direct LPS analysis. The medium from the direct experiment was collected and used to incubate another batch of MG6 for 3 h as LPS_CM analysis. mRNA levels were analyzed via qRT-PCR, normalized to the level of *Eef1a1* (elongation factor), and expressed as fold-changes relative to the LPS-only treated group. Data are presented as the mean ± S.E.M, $n = 3$; * $p < 0.05$, different letters indicated the significant differences.

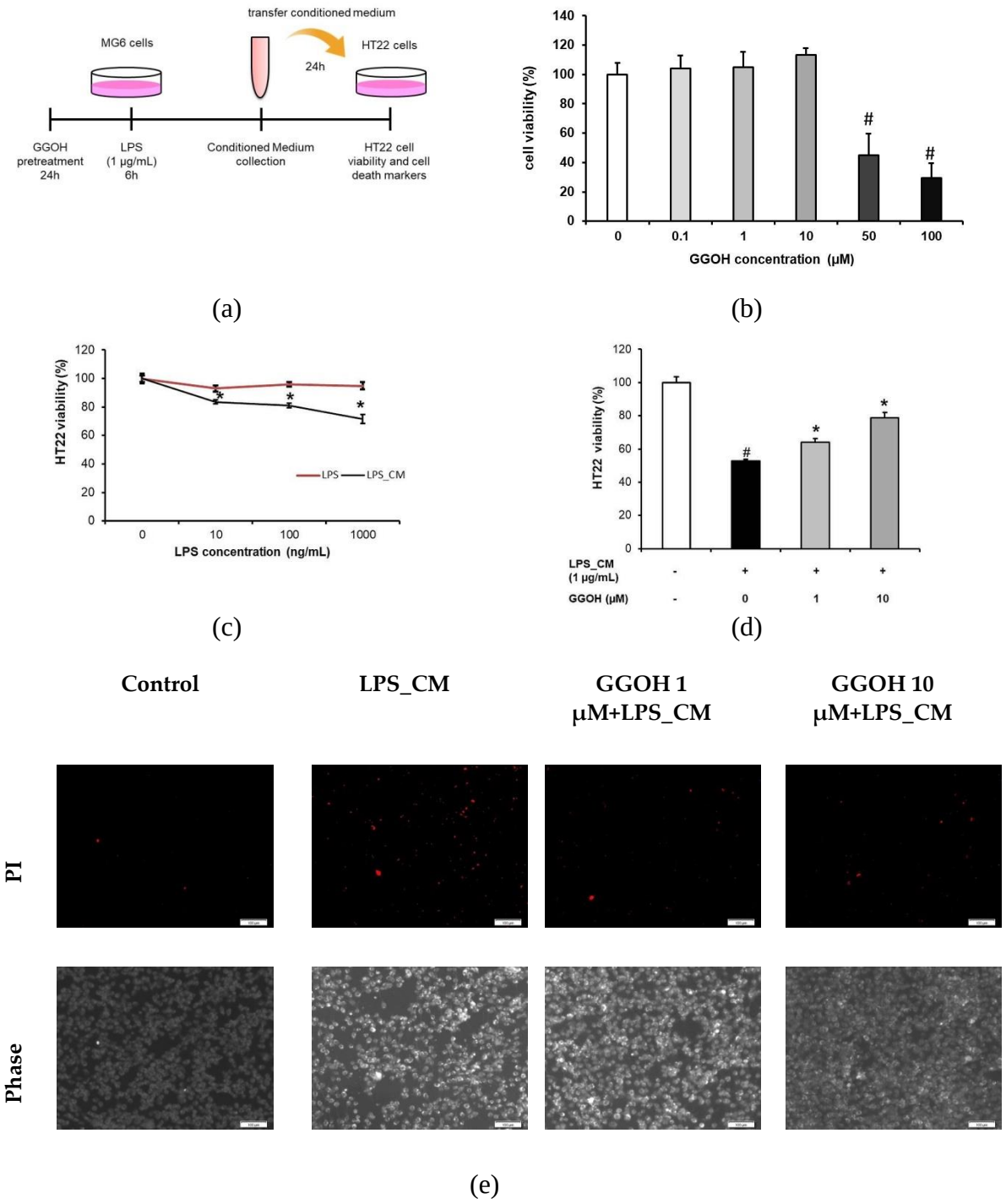
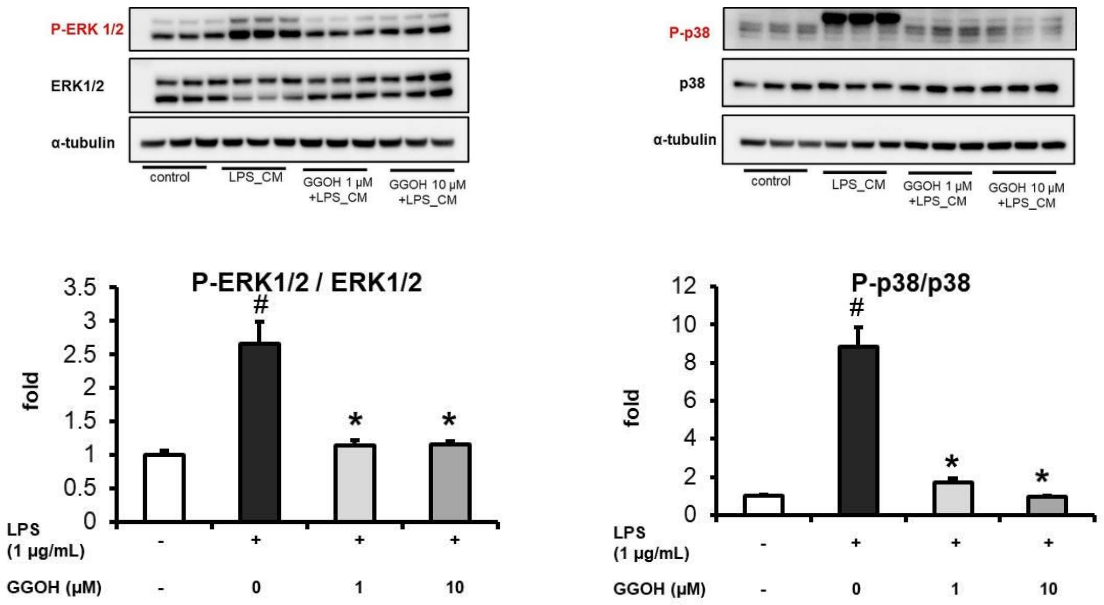


Fig. 21. The protection of HT22 cell viabilities by GGOH administration from the neurotoxicity induced by LPS-treated MG6 cells. (a) The representative conditioned-medium experiment flow; (b) HT22 cell viabilities after 24 h incubation with GGOH; (c) HT22 cell viabilities after 24 h incubation with the direct LPS and LPS_CM from MG6 cells; (d) MG6 cells were treated with LPS for 6 h, in the presence or absence of 24 h GGOH pre-treatment. The medium was then collected and used to incubate HT22 cells for the next 24 h before viability assay. Cell viabilities were evaluated using the WST-1 method; (e) Representative of PI staining and phase-contrast images of HT22 cells after incubation with LPS_CM with or without GGOH pre-treatment, scale bars 100 μm. Data are presented as the mean ± S.E.M, n = 4. # $p < 0.05$ vs untreated control. * $p < 0.05$ vs LPS_CM.



(a) (b)

Fig. 22. The western blot images and quantification of P-ERK1/2/ERK1/2 and P-p38/p38 ratios in the HT22 cells incubated with LPS_CM in the presence or absence of GGOH. Data are presented as the mean ± S.E.M, $n = 3$. # $p < 0.05$ vs untreated control. * $p < 0.05$ vs LPS_CM.

Table 1. Nucleotide sequences of primers

Gene name	Forward	Reverse
Eef1a1	GATGGCCCCAAATTCTTGAAG	GGACCATGTCAACAATTGCAG
Il-1β	CTGTGTCTTTCCCGTGGACC	CAGCTCATATGGGTCCGACA
Il-6	AGAGGAGACTTCACAGAGGATACC	AATCAGAATTGCCATTGCACAAC
Tnf-α	GACGTGGAACTGGCAGAAGAG	TCTGGAAGCCCCCCATCT
Cox-2	TGAGTACCGCAAACGCTTCT	CAGCCATTTCCTTCTCTCCTGT
Irak1	GCCAGCCAAAGAACTTGATAGAA	TACTCTGCTTGCCTTGCTCACA
Traf6	GGAATCACTTGGCACGACACTT	GGACGCAAAGCAAGGTTAACAT
iNOS	CAGAGGACCCAGAGACAAGC	TGCTGAAACATTTCCTGTGC
Arg-1	CTCCAAGCCAAAGTCCTTAGAG	AGGAGCTGTCATTAGGGACATC
Ym-1	AGAAGGGAGTTTCAAACCTGGT	GTCTTGCTCATGTGTGTAAGTGA
Socs1	CCGCCAGATGAGCCCAC	GCCAACAGACCCCAAGGAG
Bcl-3	CCTTTGATGCCCATTTACTCTA	AGCGGCTATGTTATTCTGGAC

Table 2. Antibodies used for western blot

Name	Source	Identifier
Phospho-NF-κB p65 (Ser536)	Cell Signaling Technology	#3033
NF-κB p65	Cell Signaling Technology	#8242
Phospho-IκBα (Ser32)	Cell Signaling Technology	#2859
IκBα	Cell Signaling Technology	#9242
Phospho-IKKα/β (Ser176/180)	Cell Signaling Technology	#2697
IKKβ	Cell Signaling Technology	#8943
Phospho-TAK1 (Thr184/187)	Cell Signaling Technology	#4508
TAK1	Cell Signaling Technology	#4505
IRAK1	Cell Signaling Technology	#4504
TRAF6	Funakoshi	GTX113029
α-tubulin	Sigma-Aldrich	T 5168
Lamin A/C	Cell Signaling Technology	#4777

Chapter 5

General discussion and conclusion

5.1. General Discussion

In general, we demonstrated that MK-4 and GGOH effectively modulated the LPS-induced inflammation in MG6 mouse-microglial derived cells, focusing on the NF-κB signaling cascade. MK-4 and GGOH inhibited the nuclear translocation of the NF-κB p65 subunit and maintained the IκBα protein. These two events are important in the binding of NF-κB to its recognizing site, which induces transactivation of pro-inflammatory cytokines (Chen and Greene, 2004). However, we still lacked in confirming the specific targets of MK-4 and GGOH, either on NF-κB p65 or IκBα. In the expression of phosphorylated NF-κB p65, we found that both MK-4 and GGOH had reducing effects. Although it warrants further clarification, there was the possibility that MK-4 and GGOH promoted the dephosphorylation of p65, for example, via the induction of protein phosphatase 2A (Yang et al., 2001). Another speculation is that MK-4 and GGOH encouraged the resynthesized nuclear IκBα protein, removing the binding of NF-κB p65 and terminating its transcriptional activity (Giridharan and Srinivasan, 2018; Viatour et al., 2005). The evaluation and comparison of cytoplasmic and nuclear IκBα levels after LPS administration might help to testify this idea.

In GGOH treatment, we observed that the phosphorylation of the IKK family and even more upstream regulators such as IRAK1 could be modulated. However, the previous study did not change IKK expressions after MK-4 treatment in MG6 cells (Aoyama et al., 2017). In another study using a different type of microglial cell lines induced by rotenone, MK-4 was found to inhibit the IKK phosphorylation (Yu et al., 2016). MK-4 was also found to reduce IKK expression in LPS-induced macrophage-like cells (Ohsaki et al., 2010). Therefore, it was unclear whether GGOH but not MK-4 could affect the IKK signaling event in MG6 cells. The simplest explanation is the difference in cell lines used in the experiments or different signal inducers, such as between LPS and rotenone. Another possible reason is that GGOH might undergo further post-treatment modification, for example, protein prenylation or conversion to other active metabolites (Crick et al., 1997). However, we are still uncertain considering the post-modification of MK-4 treatment. Nonetheless, both MK-4 and GGOH were suitable for inhibiting LPS-induced NF-κB activation in MG6 cells.

Another interesting observation from the current study was that MK-4 and GGOH could induce negative feedback regulators, especially *Socs1* and *Bcl-3* mRNA. These results

indicated the involvement of MK-4 and GGOH in the post-modification of NF-κB. Specifically, we found MK-4 and GGOH down-regulated the mRNA expressions of *Il-1β* and *Tnf-α*. It was linear with the up-regulation of *Bcl-3* by MK-4 and GGOH. BCL-3 is the negative regulator responsible for controlling the expression of IL-1β and TNF-α (Wessells et al., 2004). On the other hand, IL-6 expression is much controlled by another negative regulator protein, called IκB-NS (Kuwata et al., 2006). Here, we detected a lower reduction in *Il-6* mRNA expression. However, we were still unsure whether the non-association of IκB-NS caused this less reduction in Il-6 since we have not evaluated the level of this protein. Moreover, we observed that GGOH treatment induced the up-regulation of M2 markers, including *Arg-1* and *Ym-1*. The conversion of microglia to the M2 phenotype has been reported regulated by cyclic AMP (Ghosh et al., 2016). Cyclic AMP was also reported to mediate the negative regulator (Wall et al., 2009). Interestingly, in other cell lines, MK-4 and GGOH were found to induce cyclic AMP activities (Ho et al., 2019, 2018, 2016). Therefore, the MK-4 and GGOH's evaluation of cyclic AMP activity in microglial cells is urgently needed.

Given that MK-4 and GGOH similarly showed anti-inflammatory characteristics, we believed that the functional action of MK-4 came from not only the naphthoquinone ring but also from its side chain structure which is chemically similar to the isoprenoid's one. Moreover, our study using GGOH, FOH, and POH also support the hypothesa that proposes isoprenoid's inhibitory action against the NF-κB activation. Here we used an inflammation model in cell culture experiments. It gave a fast and flexible feature to understand the molecular mechanism of beneficial action from bioactive compounds. However, our study was also limited since the actual reaction in complex tissues is far complicated. Therefore, further in vivo studies such as animal experiments should be considered. Furthermore, the *in vivo* analysis to determine the safe dose of MK-4 and GGOH administrations are also essentials. Nevertheless, our current studies noticed the potential beneficial effect of MK-4 and GGOH treatment, which can provide initial clarification and an important base for neuroinflammatory modulation alternatives.

5.2. Conclusion

Here we analyzed the association of MK-4 and GGOH treatment on the attenuation of CNS inflammatory model using cell-based experiment. Using several molecular analyses, we demonstrated that :

1. MK-4 attenuated the LPS-induced inflammation in MG6 mouse microglial-derived cells via the inhibition of NF-κB p65 nuclear translocation. Moreover, MK-4 was potential to enhance the recovery of inflammation inititated by LPS possibly through the induction of negative feedback regulators.
2. GGOH also attenuated the LPS-induced inflammation in MG6 cell via the blocking of NF-κB p65 nuclear translocation and its involvement in the upstream regulator, including inhibition of IKK phosphorylation. GGOH also had a potency to direct the microglial polarization by inducing the M2 phenotype markers. In addition, other isoprenoid analogs such as FOH and POH were also promising candidates for inflammatory inhibitors.
3. In the conditioned medium experiment, the GGOH pre-treatment on the LPS-induced MG6 cells showed an indirect protective capacity by maintaining the viability of HT22 hippocampal neuron cells against the detrimental effect of microglial-mediated neurotoxicity.

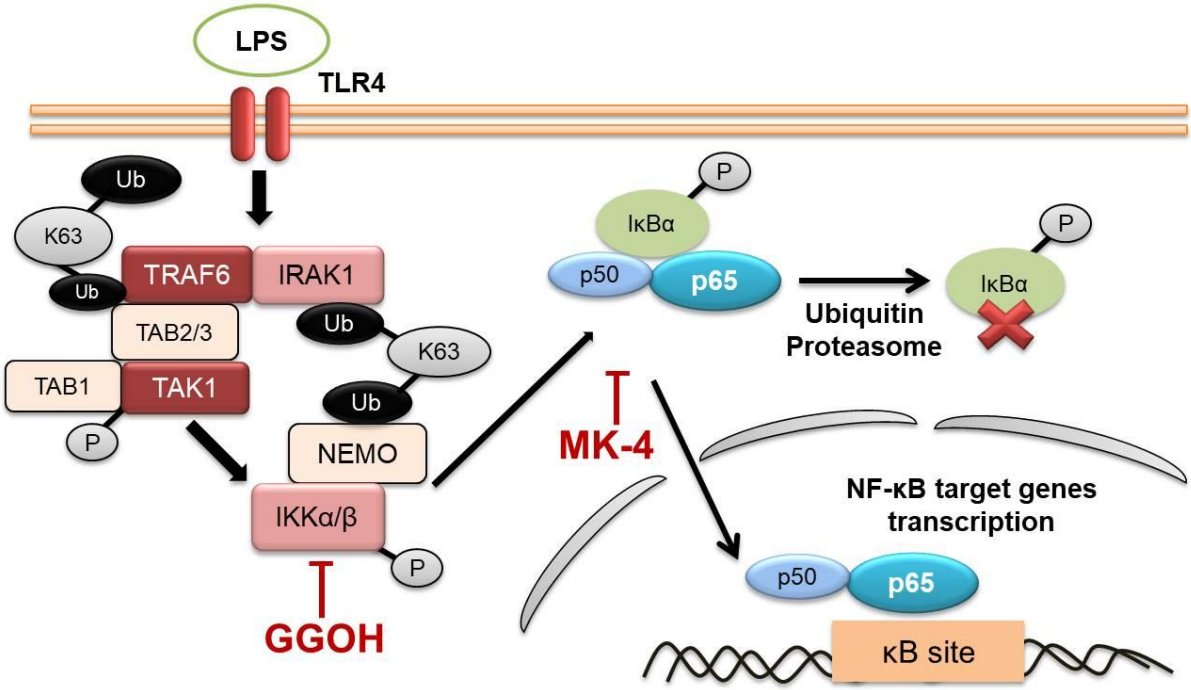


Fig. 23. The proposed mechanism of MK-4 and GGOH inhibitory action against LPS-induced inflammation in MG6 microglia derived cells. Both of MK-4 and GGOH effectively modulated the activation of NF-κB signaling cascade. MK-4 was found to block the nuclear translocation of NF-κB p65 subunit which might prevent the inflammatory cytokines transcription. On the other hand, GGOH had a potential to manage the upstream signaling by ameliorating the phosphorylation of IKKα/β which inhibited the further events. Futher studies are necessary to elucidate the specific target molecules of MK-4 and GGOH.

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