Anti-neuroinflammatory Effects of 12-Dehydrogingerdione in LPS-Activated Microglia through Inhibiting Akt/IKK/NF-κB Pathway and Activating Nrf-2/HO-1 Pathway

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Abstract
Ginger, one of worldwide consumed dietary spice, is not only famous as food supplements, but also believed to exert a variety of remarkable pharmacological activity as herbal remedies. In this study, a ginger constituent, 12-dehydrogingerdione (DHGD) was proven that has comparable anti-inflammatory activity with positive control 6-shogaol in inhibiting LPS-induced inflammation [10]. 12-DHGD may inhibit neuro-inflammation through suppressing the LPS-activated Akt/IKK/NF-κB pathway. Furthermore, 12-DHGD markedly promoted the activation of NF-E2-related factor (Nrf)-2 and heme oxygenase (HO)-1, and demonstrated that the involvement of HO-1 on the production of pro-inflammatory mediators such as NO and TNF-α by using a HO-1 inhibitor, Zinc protoporphyrin (Znpp). These results indicate that 12-DHGD may protect against neuro-inflammation by inhibiting Akt/IKK/NF-κB pathway and promoting Nrf-2/HO-1 pathway.

Key Words: Ginger, 12-Dehydrogingerdione, Neuro-inflammation, Microglial cells, Signaling pathway

INTRODUCTION
Oxidative stress and neuro-inflammation result in numerous neurodegenerative diseases of the central nervous system (CNS), such as Alzheimer disease, Parkinson’s disease, and Multiple sclerosis (Teismann et al., 2003; Infante-Duarte et al., 2008; Piirainen et al., 2017). The hallmark of neurodegenerative diseases is the persisting activation of microglia, and accumulation of pro-inflammatory factors, including NO, PGE₂, IL-6, and TNF-α (Akiyama et al., 2000). Microglia, type of neuronal support cells, play a major role in immune defense of CNS by acting as macrophages, and many non-immune tasks of brain (Glass et al., 2010). However, increasing evidence showed that uncontrolled microglia may promote neuronal cells death by secreting numerous neurotoxic factors, including NO, PGE₂, IL-6, and TNF-α, and induction of these pro-inflammatory mediators occurs in response to external stress signals through transcriptional factors, including NF-κB, AP-1, and members of the STAT family (Dendorfer et al., 1994; Glass et al., 2010; Villarino et al., 2017). For example, NF-κB, a classical signaling molecule that modulates immune response, cell survival (Mattson and Meffert, 2006), is normally sequestered in the cytosol in its IκB-bound inactivated form. When the inflammatory response occurs, the IKK complex is activated and catalyzes the phosphorylation of IκB. Then, phosphorylated IκB is degraded, and free NF-κB is released to localize to the cell nucleus and induce inflammatory gene expression (Zandi et al., 1997). Thus, inhibition of the NF-κB pathway may be a potential basis for protecting against microglial activation-related diseases in CNS.

Multiple classes of food supplements or phytochemicals have been experimentally used as effective protectors against neuro-inflammation (Choi et al., 2011). A well-studied natural dietary ingredient, ginger rhizome (Zingiber officinale Roscoe), it is not only used as edible spice in the world, but also a well-known medicinal herb due to its multiple pharmacologi-
Fig. 1. 12-DHGD does not affect cell viability, but attenuates the secretion of pro-inflammatory cytokines TNF-α and IL-6 in LPS-activated microglial cells. (A) Chemical structure of 12-DHGD (PC, 6-shogaol), which does not affect the (B) cell viability at indicated concentrations and time-points in the presence or absence of LPS, but significantly and dose-dependently inhibits the secretion of TNF-α (C) and the secretion of IL-6 (D) in LPS-activated BV-2 microglial supernatant. Values are expressed as the mean ± standard error of the mean (SEM) of at least 3 independent experiments. *p<0.05 and **p<0.001 compared to the control group; **p<0.05 and ***p<0.001 compared to the LPS group.

Fig. 2. 12-DHGD attenuates the production of NO and PGE₂ and the expression of iNOS and COX-2 in LPS-activated microglial cells. Effect of 12-DHGD (PC, 6-shogaol) on the production of NO (A) in the culture supernatant and up-regulator iNOS (B) expression in LPS-activated BV-2 and primary microglial cells. Effect of 12-DHGD on the secretion of PGE₂ (C) in the culture supernatant, up-regulator of COX-2 (D) and COX-1 (E) proteins expression, as well as mRNA expression of IL-6 and iNOS in LPS-activated BV-2 microglial cells. Values are expressed as the mean ± SEM of at least 3 independent experiments. *p<0.05, **p<0.01, and ***p<0.001 compared to the control group; *p<0.05, **p<0.001, and ***p<0.01 compared to the LPS group.

Ginger contains a lot of compounds, but its biological functions mainly attributed to pungent compounds including the 6-, 8-, and 10-series gingerols and many dehydrated analogues (Grzanna et al., 2005). These bioactive compounds share an aromatic ketone moiety with different alkyl side chain and substitution pattern on the side chain. Based on a structure-function relationship analysis, the carbon chain length plays a significant role in the antioxidant activity of the gingerols, and the unsaturated ketone moiety exhibits the more potent anti-oxidative and anti-inflammatory properties (Dugasani et al., 2010). Recently, a well-studied pungent compound, 6-shogaol, showed outstanding performance with anti-oxidative, anti-inflammatory, anti-cancer effects (Dugasani et al., 2010; Ha et al., 2012). In addition, a more recent study showed that 6 and 10-dehydro-gingerdiones from ginger also exhibit extremely high anti-oxidative and anti-inflammatory activities like 6-shogaol (Lee et al., 2012; Li et al., 2012). Thus, the other structurally similar compounds in ginger root, 12-dehydrogingerdione (DHGD), which has potentially beneficial effects, but it has not been extensively studied. To our knowledge, there is limited information pertaining to the anti-inflammatory effects of 12-DHGD in LPS-induced RAW 264.7 cells through inhibition of NO, IL-6, and PGE₂ without affecting TNF-α and IL-1β (Han et al., 2013). Moreover, the anti-inflammatory mechanism of 12-DHGD has not been elucidated, and whether 12-DHGD inhibits pro-inflammatory factors, such as TNF-α in neuro-inflammatory model remains unknown. Consequently, the objective of this study was to evaluate the anti-neuroinflammatory properties.
and mechanism of 12-DHGD in Lipopolysaccharide (LPS)-
activated microglial model.

MATERIALS AND METHODS

Chemicals

All primary and secondary antibodies were purchased from
Cell Signaling Technology (Beverly, MA, USA), including
iNOS, COX-1, COX-2, IKKα, IKKβ, p-IKKα/β, IκBα, p-IκBα, NF-κB, p-NF-κB, p-Akt (Thr308), p-Akt (Ser473), Akt, HO-1, Nrf-2, LiminB1, and GAPDH. Lipopolysaccharide (Escherichia
coli 055:B5) and other reagents were produced by Sigma-Aldrich
(St. Louis, MO, USA).

Isolation of 12-dehydroginerdione (DHGD)

Fresh gingers were collected from a greenhouse at the
herbarium of the Korea Research Institute of Chemical Tech-
ology (KRICT) and authenticated by Dr. Hong Kyung Sik of
KRICT, and a voucher specimen (KR0011) was deposited at
the herbarium of the Korea Research Institute of Chemical Tech-
ology (KRICT) Animal Care and Ethics Committee Guide-
lines. Primary microglia were isolated from the cortices of 1-
or 2 day-old C57BL/6N mouse pups as described previously
(Giulian and Baker, 1986). The brain cortex was removed and
cut into 1 mm3 fragments, and tissues were dissociated by me-
chanical trituration. The cell suspension was filtered through
µm cell strainer, and seeded into T75 culture flask in
DMEM with 10% FBS for 2 weeks. Primary microglia were col-
lected by gently shaking, and purity (>95%) was identified with
Ionized calcium binding adaptor molecule (Iba)-1 maker using
confocal fluorescence microscopy (Leica, Solms, Germany).

Quantification of NO production

BV-2 cells were seeded at 5×103 cells/well into 6-well
plates, cultured for 24 h, and treated with indicated concentra-
tions of 12-DHGD in the presence or absence of LPS (100
ng/mL) for 12 h. For measuring NO production as described
previously (Zhao et al., 2017), briefly, the culture medium was
removed, and centrifuged at 13000 rpm to eliminate cells. A
total of 50 µL of each culture medium was reacted with 50 µL
mixed Griess reagent (1% sulfanilamide, and N-(1-Naphthyl)-
Reverse transcription polymerase chain reaction (RT-PCR)
RT-PCR was performed according our previous study (Zhao et al., 2017). Briefly, Total RNA was extracted and quantified using TRizol reagent and a NanoDrop 1000 system (Thermo Fisher Scientific Inc., Lafayette, CA, USA), respectively. Then, mRNA of 5 μg was converted to cDNA using a Superscript III reverse transcriptase kit according to the manufacturer’s instructions. All target DNA bands were amplified with 2 μL of primer pairs (IL-6 sense: CACCTGCACGTCGGAGCTT and anti-sense: CCAAGCTATCTGTTAGGAGA; INF-γ sense: GACCTACAGAGGTTGGAAGG and anti-sense: TCCTTGAGGAGGCTAGGCGG; and ERK1/2 sense: GCCCATGTAGGCCAT and 2 μL of cDNA template mixed with 10 μL of Novelzyme Taq Plus Premix (Noble Bio, Suwon, Korea), then cDNA was separated by electrophoresis on 1.5% agarose gels and stained using ethidium bromide buffer for 30 min. The intensity of the PCR product was measured using an i-MAX Gel Image Analysis System (CoreBio System, Seoul, Korea).

ELISA for cytokines, IKK activity and DNA-binding assay
The culture medium was collected from 6-well plates, and centrifuged at 13000 rpm. PGE2, IL-6, and TNF-α were quantified using ELISA kits from R&D Systems Inc. (Minneapolis, MN, USA) according to the manufacturer’s instructions. Cell-free IKKα and IKKβ kinase activity was measured using IKK Kinase Enzyme System of Promega (Madison, WI, USA) in accordance with manufacturer’s protocol. DNA-binding activity was measured using NF-κB and Nrf-2 Transcription Factor Assay Kits from Abcam (Cambridge, UK) in accordance with manufacturer’s protocol.

Immunofluorescence assay
NF-κB and Nrf-2 translocation were evaluated according our previous method (Zhao et al., 2017). Briefly, cells were fixed using 3.8% p-formaldehyde for 20 min at room temperature, and the cell membranes were permeabilized by 0.5% Triton X-100 in cold PBS for 30 min and then blocked for 30 min with 5% BSA prepared in 0.1% Tween 20 (PBST). Afterward, Cells were then incubated at room temperature for 2 h in a 5% BSA solution that contained specific primary antibodies (1:100). After washing, Alexa Fluor 488-labeled IgG secondary antibody (Invitrogen, CA, USA) was added at 1:200 dilution in 5% BSA solution for 1 h, and then incubated under dark. After that, cells were stained with 25 μg/mL of 4’-6-diamidino-2-phenylindole (DAPI) in PBST, examined by confocal fluorescence microscopy.

Western blot analysis
BV-2 cells of 5×10⁵ cells/well were seeded into at 6-well plate, and cultured for 24 h, and treated with indicated concentrations of 12-DHGD and LPS (100 ng/mL) for 12 h. Cells were collected and lysed according our previous method (Zhao et al., 2017) with lysis buffer (CST) mixed 1× protease inhibitor cocktail (PIC, Roche, Penzberg, Germany) and 1× PMSF. The concentration of total protein was quantified using the Bradford reagent (Bio-Rad, Hercules, CA, USA), and purified BSA as the standard. Additionally, nuclear extract of the cells was isolated using the nuclear extract kit (Thermo Fisher Scientific Inc.) according to the manufacturer’s protocol. The protein was separated by 8% or 10% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF, Millipore, USA) membranes, which were blocked with 5% BSA and incubated with specific primary antibodies at 4°C overnight. After washing with TBST, HRP-conjugated IgG secondary antibodies were added for 1 h incubation at room temperature. The blots were determined using ECL reagents (Thermo Fisher Scientific Inc). Quantification of protein bands was analyzed using LAS4000 system (Fujifilm, Tokyo, Japan).

Statistical analysis
All numerical data are expressed as the mean ± standard error of the mean (SEM) of at least three replicates. The data were analyzed using the Graphpad Prism 7 software (GraphPad Software Inc, San Diego, CA, USA). The statistical comparisons between the different treatments were performed by one-way ANOVA with the Tukey multiple comparison test.
**RESULTS**

**Identification of 12-DHG D**

12-DHG D was identified by NMR: 1H-NMR (CDCl3, 300 MHz) δ 7.50 (1H, d, J=15.8 Hz), 7.05 (1H, d, J=8.1 Hz), 6.99 (1H, s), 6.90 (1H, d, J=8.1 Hz) 6.33 (1H, d, J=15.8 Hz, H-2), 5.61 (1H, s), 3.89 (3H, s, –OCH3), 2.35 (2H, s), 2.58 (2H, t, J=7.6 Hz, m), 1.63 (2H, m), 1.20 (16H, m), 0.87 (3H, t, J=6.6 Hz); 13C-NMR (CDCl3, 125 MHz) δ 200.2, 178.1, 147.8, 146.9, 139.9, 127.6, 123.4, 120.4, 114.9, 109.5, 100.1, 55.8 (–OCH3), 40.1, 31.9, 29.4-29.3 (6C), 25.6, 22.8, 14.1.

**12-DHG D does not affect cell viability, but attenuates TNF-α and IL-6 secretion**

Chemical structure of 12-DHGD was shown in Fig. 1A, and cell viability was measured after treatments with various concentrations and treated times of 12-DHGD in the presence or absence of LPS during 12 h. As shown in Fig. 1B, 12-DHGD in the presence and absence of LPS did not have a significant effect on microglial viability at 10 μM, while high concentrations of 12-DHGD significantly induced cytotoxicity. Subsequently, to determine the effect of 12-DHG D on neuro-inflammation responses, and maximum concentration of 10 μM was used. We determined pro-inflammatory cytokines such as TNF-α and IL-6 in LPS-activated BV-2 microglia. The secretion of both TNF-α and IL-6 in culture medium supernatant were measured using ELISA kits, and Fig. 1C and 1D showed that LPS-induced cytokines are dose-dependently suppressed by treatment with 12-DHGD. Compare to a positive control 6-shogaol, 12-DHG D is more efficient in inhibiting the secretion of TNF-α and IL-6. Compared to a previous study that TNF-α was not inhibited by treatment with 12-DHG D in RAW 264.7 cells (Han et al., 2013), the difference may depend on the cell species or treatment concentration of 12-DHG D.

**12-DHG D attenuates the production of NO and PGE2, and the expression of iNOS and COX-2**

The other pro-inflammatory mediators, including NO, PGE2, iNOS, and COX-2 were also determined in LPS-activated BV-2 or primary microglial cells. The production levels of LPS-induced NO and PGE2 in supernatants were evaluated using Griess reagents and ELISA, respectively. As shown in Fig. 2A and 2C, the production of NO and PGE2 was markedly suppressed by treatment with 12-DHG D in a dose-dependent manner. Because LPS-induced NO and PGE2 are synthesized and regulated by iNOS and COX-2, respectively, which are inducible enzymes, we determined the effects of 12-DHG D on

**Fig. 5.** 12-DHG D affects neuro-inflammation by regulating the Nrf-2/HO-1 signaling pathway in LPS-activated microglial cells. Effect of 12-DHG D on the expression of HO-1 (A) and Nrf-2 (B) in the cell nucleus (n) and cytoplasm (c), and nuclear translocation of Nrf-2 (C) (scale bar=25 μm), and (D) Nrf-2-DNA binding activity was detected by western blot, immunofluorescence, and ELISA, respectively. Effect of the 12-DHG D treatment time on the expression of Nrf-2 (E) in the absence of LPS activated BV-2 cells. Values are expressed as mean ± SEM of at least 3 independent experiments; *p<0.05, **p<0.01, and ***p<0.001 compared to the control group; #p<0.05, ##p<0.01, and ###p<0.001 compared to the LPS group.
LPS-induced iNOS and COX-2 enzymes. As shown in Fig. 2B and 2D, the expression of iNOS and COX-2 was also significantly suppressed by pretreatment with 12-DHGD. Additionally, Fig. 2A and 2B also showed that 12-DHGD inhibits NO production and iNOS expression in LPS-activated BV-2 and primary microglial cells in dose-dependent manner. To further understand the mechanism involved in the suppression of PGE2, we also investigated another up-regulator of PGE2, COX-1, which is constitutively expressed in normal tissues. As Fig. 2E showed that 12-DHGD did not inhibit the expression of COX-1 in LPS-activated BV-2 microglia. Moreover, we tried to measure the mRNA expression of IL-6 and iNOS, both of which were inhibited by treatment with 12-DHGD (Fig. 2F). These results suggest that 12-DHGD not only inhibits LPS-induced NO production, PGE2, iNOS, COX-2, and also significantly reduces mRNA expression of IL-6 and iNOS.

12-DHGD inhibits neuro-inflammation by suppressing the NF-κB signaling pathway

The NF-κB transcription factor play an important role in regulating the production of pro-inflammatory mediators and cytokines (Baeuerle and Henkel, 1994; Karin and Delhase, 2000). Thus, we investigated the effects of 12-DHGD on transcription factor NF-κB in LPS-activated BV-2 microglia. NF-κB phosphorylation in the nucleus was significantly induced by treatment of LPS, and suppressed by treatment with 12-DHGD, as shown in Fig. 3A. To visualize the translocation of NF-κB, we examined the nuclear translocation of p65-NF-κB from cytoplasm to nucleus in BV-2 cells by immunofluorescence, and found that LPS stimulated NF-κB accumulation (green fluorescence) in the nucleus, this process was clearly reversed by treatment with 12-DHGD, as shown in Fig. 3B. Additionally, because the NF-κB-DNA binding activity is essential for its basic function as a transcription factor, we measured the effect of 12-DHGD on the LPS-induced DNA-binding activity of NF-κB using a transcription activity kit (Abcam). 12-DHGD significantly reduced the NF-κB-DNA binding activity, compared to the LPS-promoted NF-κB-DNA binding activity shown in Fig. 3C. These results suggest that pre-treatment with 12-DHGD not only inhibits the phosphorylation and translocation of NF-κB, but also down-regulates the DNA binding ability of NF-κB.

12-DHGD inhibits neuro-inflammation by suppressing the Akt/IKK signaling pathway

Because the PI3K/Akt signaling pathway is necessary for efficient NF-κB activation and subsequent expression of NF-κB-regulated inflammatory genes (Sizemore et al., 1999), we examined the effect of 12-DHGD on the PI3K/Akt pathway. As shown in Fig. 4A, LPS significantly induced Akt phosphorylation (Ser473 and Thr308), but this effect was significantly suppressed by treatment with 12-DHGD. Additionally, NF-κB activation is also controlled by sequential phosphorylation, ubiquitination, and degradation of its inhibitory subunit IκB (Mercurio et al., 1997). Therefore, to establish the specific effects of 12-DHGD on regulation of NF-κB signaling pathway in LPS-activated microglial cells, we investigated two up-regulatory mechanism, IκB kinase (IKK) and IκB phosphorylation by western blot. As shown in Fig. 4B, LPS markedly increased the phosphorylation of IKKα/β, which was reduced by treatment with 12-DHGD. Furthermore, LPS-induced IκB phosphorylation and degradation were blocked by treatment with 12-DHGD as shown in Fig. 4C and 4D.

12-DHGD up-regulates Nrf-2/HO-1 key signaling pathway

In this study, we determined whether 12-DHGD induced the expression of HO-1, which is a mediator of critical cellular response against oxidative stress-induced toxicity and inflammatory responses (Syapin, 2008). As shown in Fig. 5A, compared to the LPS group, 12-DHGD significantly increased the protein expression of HO-1 in LPS-activated BV-2 microglial cells. Subsequently, we detected that up-regulator of HO-1, Nrf-2; expression of Nrf-2 was significantly induced (Fig. 5B) in both nuclear and cytoplasm, and the immunofluorescence data in Fig. 5C show that nuclear Nrf-2 is strongly increased by treatment with 12-DHGD. Furthermore, we found that 12-

Fig. 6. BV-2 cells were pre-treated with specific inhibitor LY294002 for 30 min and co-treated with 12-DHGD for 1 h, followed by co-treatment with 100 ng/ml LPS for 12 h. Phosphorylation of Akt (Thr308 and Ser473), IKK, nuclear (n) NF-κB, and TNF-α (A) was measured by western blot, and NO production (B) was measured using Griess reagents. (C) Cell-free IKKα/β and IKKβ kinase activity was measured using IKKα/β Kinase Enzyme System, respectively. 12-DHGD inhibits neuro-inflammation by up-regulating HO-1 in LPS-activated microglial cells. The effect of co-treatment with the HO-1 inhibitor (zinc protoporphyrin, Znpp) on the production of NO (D) and the secretion of TNF-α (E) in LPS-activated BV-2 microglial cells. Values are expressed as the mean ± SEM of at least 3 independent experiments; *p<0.05, **p<0.01, and ***p<0.001 compared to control or indicated group.
DHGD significantly promoted the DNA binding activity of Nrf-2, and activated the expression of Nrf-2 after 8 h in a time-dependent manner, as shown in Fig. 5D and 5E.

Confirmation on 12-DHGD affects neuro-inflammation by regulating Akt/IKK/NF-κB in LPS-induced microglial cells

To confirm the involvement of Akt in IKK/IκB/NF-κB signaling pathway, a selective inhibitor of Akt, LY294002 was used to abolish the function of Akt. As shown in Fig. 6A, LY294002 significantly inhibits Akt/IKK/IκB/NF-κB, including the phosphorylation of Akt (Ser 473 and Thr 308), the phosphorylation of IKK, and nuclear NF-κB translocation and phosphorylation. In addition, we found that LY294002 can significantly inhibit the production of NO, and more than 12-DHGD, however, co-treatment LY294002 and 12-DHGD cannot significantly reduce NO production compare to LY294002-alone treatment in Fig. 6B. Since IKK is critical kinase for activating the phosphorylation of IκB to release free NF-κB and induce inflammatory gene expression (Zandi et al., 1997). We further detected whether 12-DHGD is an IKK kinase inhibitor or not. The result as shown in Fig. 6C that 12-DHGD can significantly inhibit IKK kinase activity. The results indicate that Akt may be a key target for inhibiting inflammatory responses, and the data reveal that the LPS-induced Akt/IκB/IκBα signaling pathway is effectively suppressed by treatment with 12-DHGD, which leads to the inactivation of NF-κB in BV-2 cells.

12-DHGD attenuates neuro-inflammation by up-regulating the expression of HO-1

HO-1 plays a critical role in against the toxicity of oxidative stress and inflammatory responses (Syapin, 2008). The role of HO-1 in the 12-DHGD-mediated anti-inflammatory effect was evaluated, Zinc protoporphyrin (Znpp), HO-1 inhibitor was used for abolished the expression of HO-1. As shown in Fig. 6D and 6E; both NO and TNF-α were significantly increased after abolishing the expression of HO-1. Then, co-treatment with Znpp and 12-DHGD significantly reduced the LPS-induced NO production and TNF-α secretion. Thus, 12-DHGD-mediated HO-1 is also important signaling pathway to attenuate neuro-inflammatory effects.

DISCUSSION

Accumulating evidence suggests that over-activated microglia secrete excessive pro-inflammatory mediators, which can lead to neuro-inflammation and neurodegenerative diseases (Akiyama et al., 2000; Teismann et al., 2003; Infante-Duarte et al., 2008; Pirainnen et al., 2017). Therefore, suppression of over-activated microglia may be therapeutically valuable for treatments of neuro-inflammation and related neurodegenerative diseases. Positive control 6-Shogaol, a pungent compound in ginger, 12-DHGD, showed comparable anti-neuroinflammatory effects with 6-shogaol for inhibiting the production of pro-inflammatory mediators, including NO, IL-6, PGE2, TNF-α, iNOS, and COX-2 in LPS-activated microglial cells. It is mentioned that 12-DHGD selectively inhibits the expression of COX-2 without affecting the expression of COX-1. In addition, 12-DHGD also reduce the mRNA expression of IL-6 and iNOS, which are regulated by NF-κB.

A mechanistic study, has shown that the NF-κB transcription factor plays an important role in the production of pro-inflammatory cytokines and is believed to be a promising target for the treatment of inflammatory diseases (Tak and Firestein, 2001; Gupta et al., 2010). Thus, the effect of 12-DHGD on the NF-κB pathway was evaluated in LPS-activated BV-2 microglial cells. As Fig. 3 shown that DNA binding activity and phosphorylation of NF-κB in the nucleus were significantly stimulated by LPS, and the effects of LPS were diminished by treatment with 12-DHGD. These data were confirmed by confocal microscopy, where it was evident that 12-DHGD prevented the localization of NF-κB from the cytoplasm to the nucleus. Moreover, 12-DHGD can reduce NF-κB activation by blocking the phosphorylation and subsequent degradation of IκB in Fig. 4. Furthermore, we determined the effect of 12-DHGD on the phosphorylation of IKK; LPS-induced IKK phosphorylation was significantly inhibited by treatment with 12-DHGD. Additionally, our results raised the possibility that 12-DHGD is an IKK kinase inhibitor which consistent with a previous study that 10-DHGD directly inhibited the catalytic activity of IKK with and without the LPS-mediated induction of macrophages (Lee et al., 2012).

Furthermore, well-studied Akt is part of a signaling pathway that is necessary for inducing key immune and inflammatory responses (Ozes et al., 1999); in this study, Akt was inhibited in a dose-dependent manner by treatment with 12-DHGD. To confirm 12-DHGD-mediated attenuation of neuro-inflammation via Akt/IKK/NF-κB pathway, specific inhibitors were used to block Akt activation, which resulted in reducing phosphorylated IKK expression, nuclear NF-κB and p-NF-κB, TNF-α, and NO production. These results indicate that the Akt/IKK/NF-κB signaling pathway is an important target for inhibiting the LPS-induced neuro-inflammatory activity of 12-DHGD. Moreover, up-regulation of HO-1 expression is an adaptive and protective response to oxidative injury (Syapin, 2008; Jeong et al., 2016). In this study, we found that 12-DHGD significantly up-regulates the expression of HO-1 in Fig 6. Since previous data have suggested an anti-inflammatory function for Nrf-2, which can inhibit the NF-κB activation and up-regulate HO-1 (Cuadrado et al., 2014). In this study, we detected that 12-DHGD also induces the activation of Nrf-2 in a dose- and time-manner in BV-2 microglial cells. A subsequent mechanistic evaluation showed that 12-DHGD-induced HO-1 significantly contributed to the inhibition of NO and TNF-α by abolishing HO-1 expression. Although PI3K/Akt signaling pathway has been reported to involve in inflammatory and oxidative process via activating both NF-κB and Nrf-2 in various cells, respectively (Wang et al., 2008; Bai et al., 2009). It is interesting that 12-DHGD inhibits the activation of Akt/NF-κB signaling pathway, instead increases Nrf-2/HO-1 expression. Similar to 12-DHGD, there are many famous natural products show similar effects in immune cell, such as curcumin, morin, and schisandrin A (Cianciulli et al., 2016; Jung et al., 2017; Kwon et al., 2018). The main reason may due to difference of cells, and further study would be required.

In conclusion, this study demonstrates that 12-DHGD has considerable anti-neuroinflammatory effects toward inhibiting the production of pro-inflammatory mediators, such as IL-6, TNF-α, PGE2, NO, iNOS, and COX-2. The molecular mechanism by which 12-DHGD inhibits neuro-inflammatory responses in microglia is based on 12-DHGD-mediated the regulation
of inflammation-related signaling pathways, including inhibition of Akt/IKK/NF-κB, and activation of Nrf-2/HO-1 pathways.

CONFLICT OF INTEREST

The authors have no conflicts of interest.

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