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A kavalactone derivative inhibits lipopolysaccharide-stimulated iNOS induction and NO production through activation of Nrf2 signaling in BV2 microglial cells

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ABSTRACT

Neuroinflammation and oxidative stress are involved in the pathogenesis of neurodegenerative diseases such as Alzheimer's diseases and Parkinson's disease. Naturally derived kavalactones isolated from *Piper methysticum* (Piperaceae) have been shown to exhibit neuroprotective effects. We have previously reported that a chemically synthesized kavalactone derivative, 2′,6′-dichloro-5-methoxymethyl-5,6-dehydrokawain (compound 1) protects against oxidative stress-induced neuronal cell death through activation of Nrf2 signaling. In the present study, we examined the effect of compound 1 on neuroinflammation. In BV2 microglial cells, compound 1 strongly inhibited LPS-stimulated iNOS induction and NO production, but did not affect LPS-stimulated induction of COX2. At 6 h after LPS challenge, when iNOS induction was not clearly seen, treatment with LPS or compound 1 alone increased expression of heme oxygenase 1 (HO-1) whose transcription is regulated by Nrf2. When treated with both, compound 1 enhanced LPS-stimulated HO-1 induction, which was more evident at 24 h after LPS treatment. Furthermore, LPS-stimulated activation of Nrf2 signaling and nuclear translocation of Nrf2 were potentiated by compound 1. The mechanism by which compound 1 activated Nrf2 signaling was supposed to be a covalent modification of the sulfhydryl groups of Keap1 by an α,β-unsaturated carbonyl group present in the compound 1. Treatment with hemin, a HO-1 inducer, and with $[Ru(CO)_3Cl_2]_2$, a CO donor, decreased LPS-stimulated iNOS induction and NO production. In contrast, siRNA-mediated knockdown of HO-1 expression reduced the inhibitory effect of compound 1 on LPS-stimulated iNOS induction and NO production. The compound 1 inhibited LPS-stimulated ERK phosphorylation after LPS treatment. Finally, compound 1 suppressed LPS/IFN-γ-stimulated NO production in primary microglial cells. These results suggest that compound 1 is capable of inhibiting LPS-stimulated iNOS induction and NO production via activation of Nrf2 signaling and HO-1 induction in microglial cells. Taken together, compound 1 has a potential to reduce neuroinflammation as well as oxidative stress in neurodegenerative diseases through activation of Nrf2 signaling.

1. Introduction

Microglial activation and neuroinflammation as well as oxidative stress are implicated in a variety of neurodegenerative diseases including Alzheimer's disease and Parkinson's disease [1]. Pharmacological or nutritional intervention to attenuate these processes has a potential to alleviate neurodegenerative diseases.

Transcription factor Nrf2 (nuclear factor E2-related factor 2) is a master regulator of detoxifying/antioxidant phase II enzymes including heme oxygenase 1 (HO-1). Nrf2 binds to a specific DNA sequence termed antioxidant response element (ARE) present in

Abbreviations: Nrf2, nuclear factor E2-related factor 2; HO-1, heme oxygenase-1; Keap1, kelch-like ECH-associated protein 1; CO, carbon oxide; TLR4, toll like receptor 4; NO, nitric oxide; iNOS, inducible isoform of nitric oxide synthase; LPS, lipopolysaccharide; IFN-γ, interferon-γ.

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the promoter region of phase II enzymes and enhances their transcription [2,3]. Under unstressed conditions, Nrf2 associates with a cytoplasmic repressor Keap1 (kelch-like ECH-associated protein 1) and undergoes proteasome-dependent degradation. Covalent or oxidative modification of the cysteine sulfhydryl groups of Keap1 by electrophiles or reactive oxygen species (ROS), respectively, leads to conformational changes of Keap1, resulting in nuclear translocation of Nrf2. Nuclear translocation is also enhanced by phosphorylation of Nrf2 by protein kinases [4]. Naturally derived compounds such as curcumin and sulforaphane interact with Keap1 and activate Nrf2 signaling [5,6]. Accumulating evidence has demonstrated that activation of Nrf2 signaling attenuates oxidative stress in neurodegenerative diseases through induction of phase II antioxidant enzymes such as HO-1 [7]. On the other hand, it has been also reported that Nrf2 activation and HO-1 induction are involved in the regulation of inflammation [8]. In macrophages and glial cells, HO-1 induction caused by LPS stimulation results in production of carbon monooxide (CO), which in turn inhibits LPS signaling [9]. In macrophages, the HO-1/CO pathway suppresses LPS signaling by regulating the interaction of toll like receptor 4 (TLR4) with caveolin-1 [10]. Furthermore, naturally derived compounds have been shown to suppress LPS signaling via HO-1 induction in glial cells [11–13].

Piper methysticum popularly known as Kava belongs to the pepper family (Piperaceae) [14]. Kava beverage is produced by water extraction of roots, which has been traditionally used in social and ceremonial events and consumed in the South Pacific Islands. Kava extracts have anxiolytic, sedative and analgesic effects, and most of these pharmacological effects have been ascribed to six kavalactones isolated from kava extracts including yangonin, kawain and methysticin [15]. Medicines and supplements containing kava extracts had been used worldwide, but were withdrawn from the markets due to hepatotoxicity [16,17]. The hepatotoxicity, however, was later suggested to be caused by contamination with alkaloids [18]. A recent clinical trial demonstrated that the water extracts of kava exhibit significant anxiolytic and anti-depressant activities with no safety concerns [19].

The administration of naturally derived kavalactones to mice ameliorated cerebral infarction [20]. It has been reported that naturally derived kavalactones attenuate cell death caused by amyloid β via activation of Nrf2 signaling in rat pheochromocytoma PC12 cells, a model of neuronal cells [21]. In an attempt to identify a more potent Nrf2 activator, we synthesized a series of chemically modified kavalactones and found that 2′,6′-dichloro-5-methoxymethyl-5,6-dehydrokawain [(E)-6-(2′,6′-dichlorostyryl)-4-methoxy-5-(methoxymethyl)-2H-pyran-2-one] (hereinafter referred to as compound 1) activates Nrf2 signaling more strongly than naturally derived compounds in PC12 cells [22]. The compound 1 ameliorated H_2O_2-induced PC12 cell death through activation of Nrf2 signaling and induction of phase II antioxidant enzymes including HO-1. Thus, we and others have demonstrated that compound 1 and naturally derived kavalactones are effective in neuroprotection in part through reduction of oxidative stress via activation of Nrf2 signaling.

Nitric oxide (NO) is involved in a variety of important physiological processes including vasodilatation, neurotransmission and host defense [23]. However, an excessive amount of NO is detrimental, resulting in inflammatory diseases such as neurodegenerative diseases as well as rheumatoid arthritis, gastritis, bowel inflammation and bronchitis [24]. In macrophages and microglial cells, NO is synthesized by inducible isoform of nitric oxide synthase (iNOS), which catalyzes the reaction of L-arginine to L-citrulline and NO. The iNOS expression is induced in response to various stimuli such as lipopolysaccharide (LPS), interferon, tumor necrosis factor α and interleukin 1β [25].

In the present study, we investigated if the chemically synthesized kavalactone derivative, compound 1, could attenuate neuroinflammation in addition to oxidative stress. In microglial BV2 cells, we examined the effect of compound 1 on LPS-stimulated iNOS induction and NO production and explored the underlying mechanisms with a special focus on Nrf2 signaling and HO-1 induction. We finally studied if compound 1 could inhibit LPS/interferon-γ (IFN-γ)-stimulated NO production in primary microglial cells similarly in BV2 cells.

2. Materials and methods

2.1. Antibodies

The antibodies recognizing Akt, p-Akt, ERK1/2, p-ERK1/2, p38, p-p38, iNOS, COX2, NFκB and lamin A/C were purchased from Cell Signaling Technology (Beverly, CA, USA). Anti-HO-1 and -β-actin antibodies were from Stressgen Bioreagents (Ann Arbor, MI, USA) and Sigma–Aldrich (St. Louis, MO, USA), respectively. Antibody against Nrf2 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.2. A chemically synthesized kavalactone derivative

The kavalactone derivative, compound 1, 2′,6′-dichloro-5-methoxymethyl-5,6-dehydrokawain [(E)-6-(2′,6′-dichlorostyryl)-4-methoxy-5-(methoxymethyl)-2H-pyran-2-one] was chemically synthesized and its chemical structure was confirmed by nuclear magnetic resonance (NMR) and mass spectrometric analyses as previously described [22].

2.3. BV2 cell culture and treatments

The murine BV2 microglial cell line was obtained from Dr. Makoto Sawada (Nagoya University, Nagoya, Japan). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml of penicillin and 100 μg/ml streptomycin in a humidified atmosphere of 5% CO_2 at 37 °C. Cells were seeded in 6-well plates at a density of 4×10^5 cells/well. Cells were treated with LPS (Sigma–Aldrich) at 1 μg/ml. An NO donor, S-nitroso-N-acetyl-DL-penicillamine (SNAP), a HO-1 inducer, ferriprotoporphyrin IX chloride (hemin), and a CO donor, tricarbonyldichlororuthenium ($[Ru(CO)_3Cl_2]_2$), were obtained from Wako Pure Chemical Industries (Osaka, Japan), Frontier Scientific (Logan, UT, USA) and Sigma–Aldrich, respectively.

2.4. Primary microglial cell culture and treatments

Microglial cells were isolated from the mixed cultures of cerebrocortical cells from postnatal day 1–2 C57/BL6 mice, as described previously [26]. In brief, cortical tissue was trypsinized for 3 min, dissociated with a fire-polished pipette. Mixed glial cells were cultured for 9–12 day in DMEM (Nissui, Tokyo, Japan) supplemented with 10% FBS (Hyclone Labratories, Logan, UT, USA), 2 mM L-glutamine, 0.2% D-glucose, 5 μg/ml insulin, 0.37% $NaHCO_3$, 100 U/ml penicillin, 100 μg/ml streptomycin, with medium changes every 5 day. Microglial cells were then separated from the underlying astrocytic layer by gentle shaking of the flask for 2 h at 37 °C in a shaker-incubator (120 rpm). Microglial cells were then isolated as strongly adhering cells after unattached cells were removed. The cells were plated in 24-well plates (8×10^5 cells/dish). The purity of microglia was >98%, which was evaluated by staining with Iba-1, a marker for microglia/macrophage. Cells were treated with LPS

(Sigma–Aldrich) at 200 ng/ml and IFN-γ (Pepro Tech, Rocky Hill, NJ, USA) at 25 ng/ml.

2.5. Measurement of nitrite production

The nitrite accumulated in the culture medium was measured as an indicator of NO production using the Griess reagent kit (Promega, Madison, WI, USA). Cell culture media were centrifuged at 4 °C for 5 min and the supernatant was subjected to measurement of nitrite.

2.6. Western blot analysis

Whole cell extracts were prepared using the RIPA buffer (10 mM Tris–HCl [pH 7.5], 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.1% SDS, 0.1% sodium deoxycholate) containing protease inhibitor cocktails and phosphatase inhibitor cocktails 1 and 2 (Sigma–Aldrich). Nuclear and cytoplasmic fractions were isolated using the NE-PER nuclear and cytoplasmic extraction reagents (Thermo Fisher Scientific, San Jose, CA, USA). Extracts were separated by SDS-PAGE and transferred onto PVDF membranes. After blocking with 5% skim milk for 1 h, membranes were incubated overnight at 4 °C with a primary antibody and then reacted with a HRP-conjugated secondary antibody for 1 h. Immunoreactive proteins were visualized using the ECL Prime Western blotting detection reagents (GE Healthcare, Piscataway, NJ, USA) and the LAS 4000 imaging system (Fuji Film, Tokyo, Japan). Quantitative analysis of band intensities was performed using the Multi Gauge software (Fuji Film). Densitometric data of Western blots from three independent experiments were used for statistical analysis.

2.7. Transfection and luciferase assay

The pGL4 vector (Promega) containing ten copies of the antioxidant response element (ARE) enhancer sequences present within the promoter region of glutathione S-transferase Ya subunit gene [27] upstream of the minimal promoter fused to a firefly luciferase gene was used as an ARE reporter gene. The ARE firefly luciferase reporter vector and the Renilla luciferase expression vector (phRL; Promega) were transfected into cells by using lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA). Luciferase activities were measured using the Pikkagene Dual luciferase assay system (TOYO B-Net, Tokyo, Japan). Firefly luciferase activity was normalized to Renilla luciferase activity.

2.8. Small interfering RNA transfection

Small interfering RNA (siRNA) for mouse HO-1 was purchased from Thermo Scientific Dharmacon (Lafayette, CO, USA). Negative Control Medium GC Duplex #2 obtained from Invitrogen was used as a negative control siRNA. The siRNAs were transfected into cells by using lipofectamine 2000 reagent according to the manufacturer's protocol (Invitrogen).

2.9. Statistical analysis

All data were analyzed using one-way ANOVA followed by Fisher's LSD test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Compound 1 and naturally derived kavalactones reduce LPS-stimulated iNOS induction and NO production in BV2 microglial cells

Treatment for 24 h with compound 1 and naturally derived kavalactones (yangonin, kawain and methysticin) (Suppl. Fig. 1) did not affect the viability of BV2 microglial cells at a concentration of 10 μM (data not shown). We therefore investigated their effects on LPS-stimulated iNOS induction and NO production at 10 μM in BV2 cells. As shown in Fig. 1, pretreatment for 1 h with compound 1 as well as yangonin, kawain and methysticin resulted in an inhibition of LPS-stimulated NO production. The inhibitory effect of compound 1 was much stronger than those of naturally derived kavalactones. Consistent with these findings, iNOS induction at 24 h after LPS challenge was suppressed more effectively by compound 1 than by yangonin, kawain and methysticin. However, compound 1 and naturally derived kavalactones did not affect LPS-stimulated cyclooxygenase 2 (COX2) induction. These results suggest that both compound 1 and naturally derived kavalactones are capable of reducing iNOS induction and NO production in LPS-stimulated BV-2 cells.

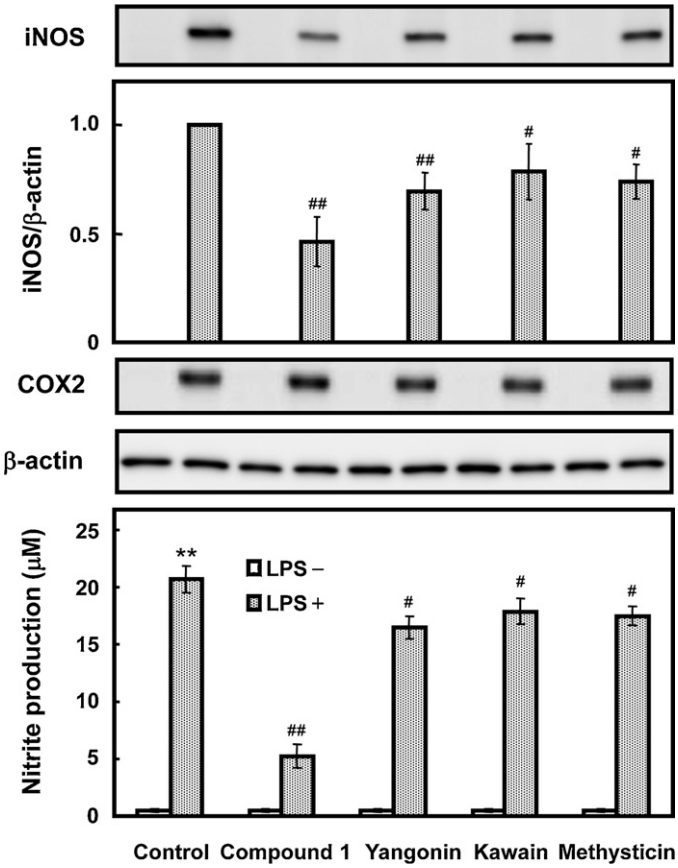

Fig. 1. Effects of compound 1 and naturally derived kavalactones on LPS-stimulated iNOS induction and NO production in BV2 cells. BV2 cells were incubated for 1 h in the absence (control) or presence of 10 μM compound 1 and naturally derived kavalactones (yangonin, kawain and methysticin), and then treated with or without LPS (1 μg/ml). Twenty-four hours after LPS treatment, cell lysates were harvested and subjected to Western blot analysis for iNOS, COX2 and β-actin. A representative blot from three independent experiments is shown along with densitometric data (mean ± SE, $n = 3$). The band intensities were normalized by β-actin and the data were presented as a ratio relative to control with LPS. Cell culture media were also harvested for measurement of nitrite (mean ± SE, $n = 3$). $^{**}P < 0.01$ vs. control without LPS, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. control with LPS.

Supplementary material related to this article found, in the online version, at http://dx.doi.org/10.1016/j.phrs.2013.02.002.

3.2. Compound 1 inhibits LPS-stimulated iNOS induction and NO production in BV2 cells in a concentration-dependent manner

Since compound 1 was more effective than naturally derived kavalactones in inhibiting LPS-stimulated iNOS induction and

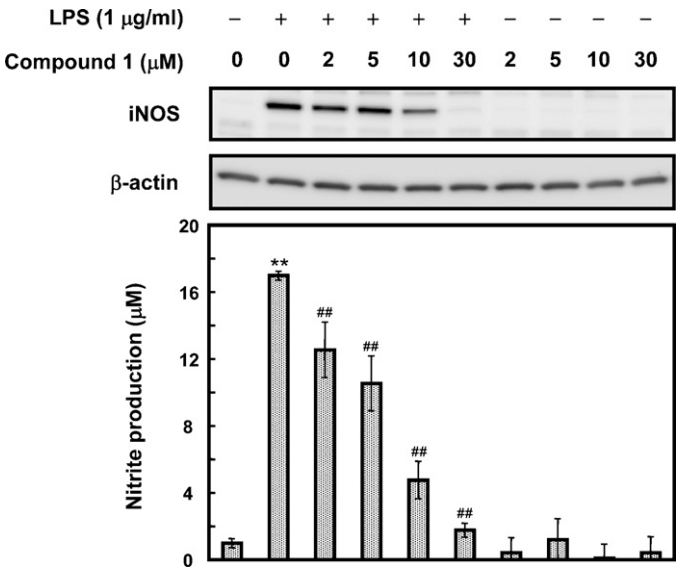

Fig. 2. Effects of treatment with increasing concentrations of compound 1 on LPS-stimulated iNOS induction and NO production in BV2 cells. BV2 cells were incubated for 1 h in the absence or presence of increasing concentrations of compound 1 (2, 5, 10 and 30 μM), and then treated with or without LPS (1 μg/ml). Twenty-four hours after LPS treatment, cell lysates were harvested and subjected to Western blot analysis for iNOS and β-actin. A representative blot from three independent experiments is shown. Cell culture media were also harvested for measurement of nitrite (mean ± SE, $n = 3$). $^{**}P < 0.01$ vs. cells without compound 1 and without LPS, $^{\#\#}P < 0.01$ vs. cells without compound 1 and with LPS.

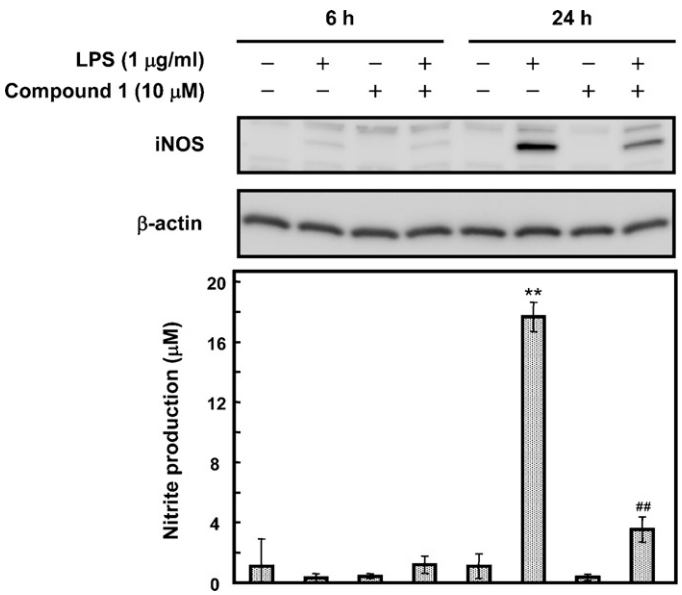

Fig. 3. Effects of time of treatment with compound 1 on LPS-stimulated iNOS induction and NO production in BV2 cells. BV2 cells were incubated for 1 h in the absence or presence of 10 μM compound 1, and then treated with or without LPS (1 μg/ml). Six and 24 h after LPS treatment, cell lysates were harvested and subjected to Western blot analysis for iNOS and β-actin. A representative blot from three independent experiments is shown. Cell culture media were also harvested for measurement of nitrite (mean ± SE, $n = 3$). $^{**}P < 0.01$ vs. cells without compound 1 and without LPS, $^{\#\#}P < 0.01$ vs. cells without compound 1 and with LPS.

NO production (Fig. 1), we focused on compound 1 in the subsequent experiments. We treated BV2 cells for 1 h with increasing concentrations of compound 1 (0, 2, 5, 10 and 30 μM) prior to LPS challenge. The results showed that compound 1 inhibits LPS-stimulated iNOS induction and NO production in a concentration-dependent manner (Fig. 2).

3.3. Induction of iNOS and production of NO are not clearly seen 6 h after treatment with LPS in BV2 cells

We examined if the inhibitory effect of compound 1 on iNOS induction and NO production is seen earlier than 24 h in LPS-stimulated BV2 cells. At 6 h after LPS treatment, iNOS expression was not clearly seen compared with that at 24 h (Fig. 3). In support of this finding, NO production was not at all elevated at 6 h after LPS challenge, indicating that it takes more than 6 h before LPS-stimulated iNOS induction and subsequent NO production become apparent in BV2 cells.

3.4. Pretreatment with compound 1 potentiates LPS-stimulated HO-1 induction in BV2 cells

It has been shown that LPS enhances HO-1 expression in macrophages [8,9]. In BV2 cells, HO-1 expression was increased by 1.4 fold at 6 h after LPS treatment and by 3.0 fold at 24 h (Fig. 4). Treatment with compound 1 alone resulted in an increase in HO-1 expression at 6 h (1.6 fold) and 24 h (1.7 fold). When treated with both, compound 1 enhanced LPS-stimulated HO-1 induction at both 6 and 24 h (2.6 and 5.1 fold, respectively). These results suggest that compound 1 is capable of potentiating LPS-stimulated HO-1 induction in BV2 cells. It is important to note that HO-1 expression was increased in BV2 cells at 6 h after LPS treatment, when iNOS protein induction was not clearly seen (Fig. 3).

3.5. Nuclear translocation of Nrf2 and activation of Nrf2 signaling caused by LPS are enhanced by pretreatment with compound 1

Since expression of phase II enzymes including HO-1 is regulated by Nrf2 at the transcription level, we examined the effects of LPS, compound 1 and both on nuclear translocation of Nrf2 in BV2 cells. After treatment with compound 1, LPS and both, Nrf2 nuclear translocation was evident after 60 min (Fig. 5A). At 120 min, treatment with LPS, compound 1 and both increased

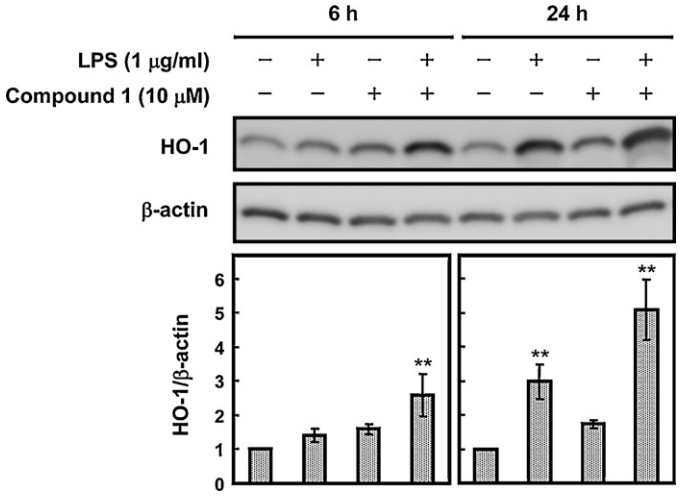

Fig. 4. Effects of compound 1 on LPS-stimulated HO-1 induction in BV2 cells. BV2 cells were incubated for 1 h in the absence or presence of 10 μM compound 1, and then treated with or without LPS (1 μg/ml). Six and 24 h later, cell lysates were harvested and subjected to Western blot analysis for HO-1 and β-actin. A representative blot from three independent experiments is shown along with densitometric data (mean ± SE, $n = 3$). The band intensities were normalized by β-actin and the data were presented as a ratio relative to cells without compound 1 and without LPS $^{**}P < 0.01$ vs. cells without compound 1 and without LPS.

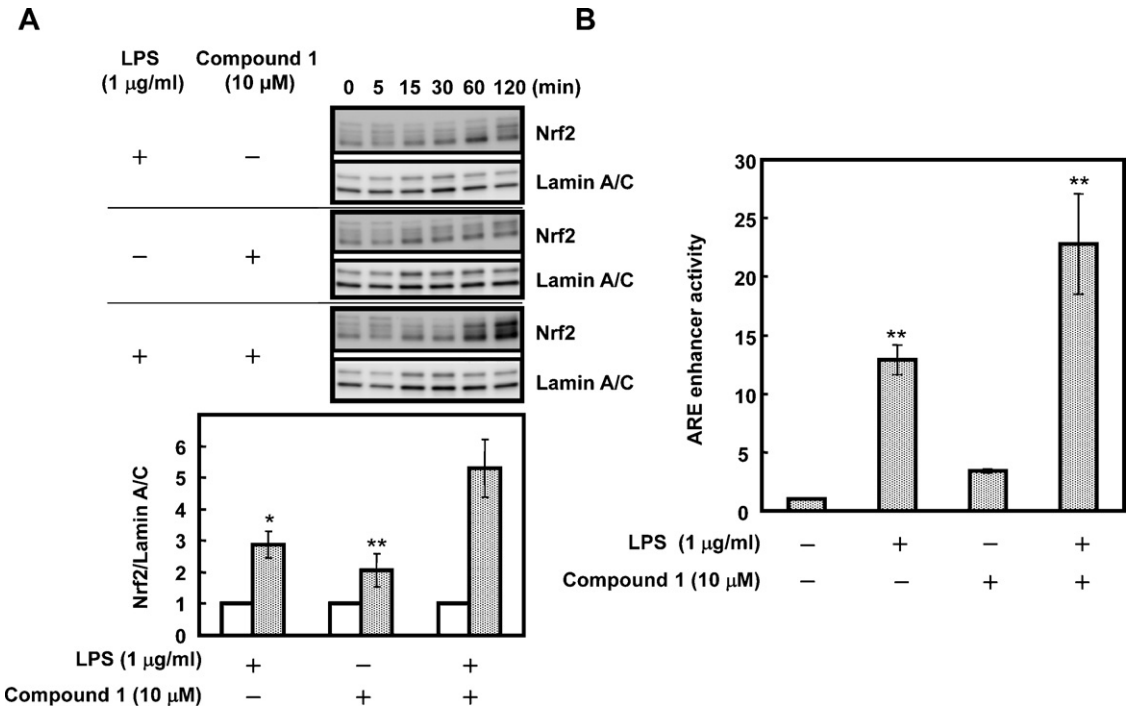

Fig. 5. Effects of compound 1 on Nrf2 nuclear translocation and Nrf2 signaling in LPS-stimulated BV2 cells. (A) BV2 cells were incubated for 1 h in the absence or presence of 10 μM compound 1, and then treated with or without LPS (1 μg/ml). At indicated time points, nuclear fractions were harvested and subjected to Western blot analysis for Nrf2 and lamin A/C, a nuclear marker. A representative blot from three independent experiments is shown along with densitometric data (mean ± SE, $n = 3$). The band intensities were normalized by lamin A/C and the data were presented as a ratio of Nrf2 at 120 min to that at 0 min. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$ vs. cells with both LPS and compound 1. (B) BV2 cells were transfected with the ARE firefly luciferase reporter vector and Renilla luciferase control vector. Twenty-four hours after transfection, cells were incubated for 1 h in the absence or presence of 10 μM compound 1, and then treated with or without LPS (1 μg/ml) for 24 h, followed by luciferase assays. The firefly luciferase activity was normalized to the Renilla luciferase activity. The ARE enhancer activity was shown as a ratio to cells without compound 1 and without LPS (mean ± SE; $n = 3$; $^{**}P \leq 0.01$).

Nrf2 nuclear translocation by 2.9, 2.1 and 5.3 fold, respectively, as compared to the basal level at 0 min. In addition, we performed luciferase reporter assay to evaluate the effects of treatments on Nrf2 signaling. LPS and compound 1 increased the ARE (antioxidant response element) enhancer activity by 12 fold and 3 fold, respectively. When treated with both, the enhancer activity was synergistically increased by 23 fold (Fig. 5B). These results indicate that compound 1 activates Nrf2 signaling by itself and also potentiates LPS-stimulated Nrf2 signaling. Combined with the previous data (Fig. 4), it is suggested that compound 1 is capable of enhancing LPS-stimulated activation of Nrf2 signaling and induction of HO-1.

3.6. *HO-1 overexpression by treatment with hemin inhibits LPS-stimulated iNOS induction and NO production in BV2 cells*

HO-1 has been shown to inhibit LPS-stimulated iNOS induction in macrophages [28]. We thus analyzed the effects of HO-1 overexpression on LPS-stimulated iNOS induction and NO production using hemin, a strong inducer of HO-1 expression. We treated cells with increasing concentrations of hemin (0, 20, 50 and 100 μM) in the absence of LPS and confirmed that hemin indeed induced HO-1 expression in BV2 cells (Fig. 6). When treated with LPS alone, HO-1 expression was increased by 2.3 fold as observed before in Fig. 4, which was significantly upregulated by treatment with hemin. Hemin treatment markedly inhibited LPS-stimulated iNOS induction even at 20 μM and the inhibitory effect was most prominent at 100 μM. Consistent with these findings, hemin treatment strongly suppressed NO production in a concentration-dependent manner. These results suggest that HO-1 inhibits LPS-stimulated iNOS induction and NO production in BV2 cells.

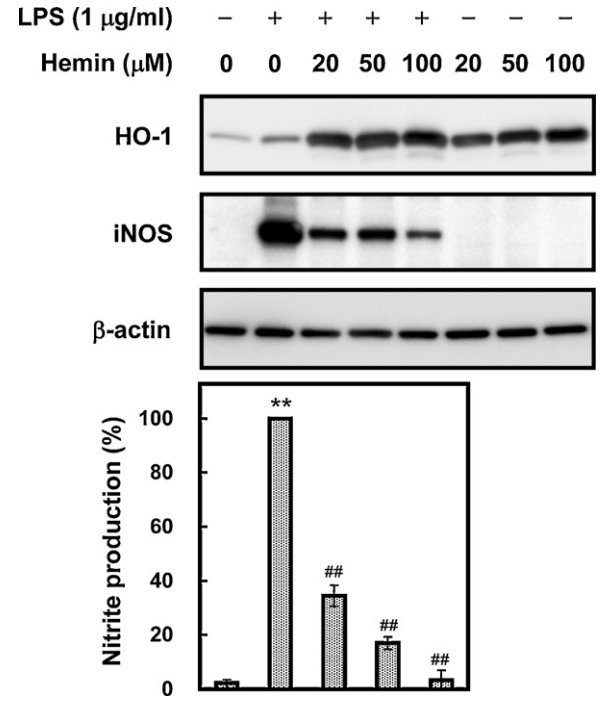

Fig. 6. Effects of treatment with hemin on LPS-stimulated iNOS induction and NO production in BV2 cells. BV2 cells were treated with or without LPS (1 μg/ml) in the absence or presence of hemin, an HO-1 inducer (20, 50 and 100 μM). Twenty-four hours later, cell lysates were harvested and subjected to Western blot analysis for HO-1, iNOS and β-actin. A representative blot from three independent experiments is shown. Cell culture media were also harvested for measurement of nitrite (mean ± SE, $n = 3$). $^{**}P < 0.01$ vs. cells without compound 1 and without LPS, $^{\#\#}P < 0.01$ vs. cells without compound 1 and with LPS.

3.7. Treatment with $[Ru(CO)_3Cl_2]_2$ inhibits LPS-stimulated iNOS induction and NO production in BV2 cells

HO-1 catalyzes the oxidative degradation of heme to free iron, biliverdin and CO [29]. The HO-1/CO system has been shown to be involved in a variety of cellular processes including inflammation. Here, we examined if CO could inhibit LPS-stimulated iNOS induction and NO production in BV2 cells. Treatment with increasing concentrations of $[Ru(CO)_3Cl_2]_2$, a CO donor (0, 10 and 30 μM), did not affect the expression level of HO-1 in LPS-stimulated BV2 cells, but the CO donor decreased LPS-stimulated iNOS induction and NO production at 30 μM (Fig. 7), suggesting that CO generated by HO-1 may participate in the inhibition.

3.8. Treatment with S-nitroso-N-acetyl-DL-penicillamine (SNAP) induces HO-1 expression in BV2 cells

It has been previously reported that NO activates Nrf2 signaling by S-nitrosylation of Keap1 in PC12 cells [30]. We thus analyzed the effect on HO-1 expression of SNAP, an NO donor. As shown in Fig. 8, SNAP significantly increased HO-1 expression as well as NO production at 100 μM or higher. These results together with the previous data (Figs. 6 and 7) suggest that NO produced by activation of LPS signaling and subsequent induction of iNOS expression activates Nrf2 signaling and induces HO-1 expression, resulting in inhibition of LPS-stimulated iNOS induction and NO production in part via CO production.

3.9. Knockdown of HO-1 expression by siRNA reduces the inhibitory effects of compound 1 on LPS-stimulated iNOS induction and NO production in BV2 cells

In an attempt to demonstrate the direct role of HO-1 in the inhibition by compound 1 of LPS-stimulated iNOS induction and NO production, we transfected BV2 cells with a control siRNA or HO-1 siRNA and then treated cells with or without LPS and with or without compound 1. HO-1 expression was effectively reduced by siRNA transfection (Fig. 9). The inhibitory effect of compound 1 on LPS-stimulated iNOS induction was decreased in HO-1 siRNA-transfected cells (0.55 fold) compared to control siRNA-transfected cells (0.24 fold). Similarly, HO-1 knockdown reduced the inhibitory effect of compound 1 on LPS-stimulated NO production from 0.05 fold to 0.71 fold. These results suggest that HO-1 plays a direct role in the inhibition of LPS-stimulated iNOS induction and NO production by compound 1.

3.10. Compound 1 inhibits LPS-stimulated ERK phosphorylation in BV2 cells

LPS binding to the cell surface receptor triggers activation of TLR4 and the downstream signaling molecules including IκB and mitogen-activated protein kinases such as c-Jun NH2-terminal protein kinase (JNK), p38 MAP kinase and extracellular signal-regulated kinase (ERK). LPS signaling activates transcription factors such as nuclear factor kappa B (NFκB) and activator protein 1 (AP1), culminating in the expression of pro-inflammatory genes including iNOS and COX2 [31,32]. Here, we investigated the effects of compound 1 on intracellular signal transduction. Treatment with compound 1 alone did not affect phosphorylation of p38, JNK and Akt, but inhibited that of ERK (Fig. 10A). On the other hand, compound 1 significantly inhibited ERK phosphorylation at 15 min after LPS treatment (x0.56 fold, $P < 0.01$ vs. without compound 1), but did not affect JNK and Akt phosphorylation (Fig. 10B). The nuclear translocation of NFκB induced by LPS stimulation was not significantly different between compound 1-treated and untreated cells. Our results suggest that the ERK but not NFκB pathway may be

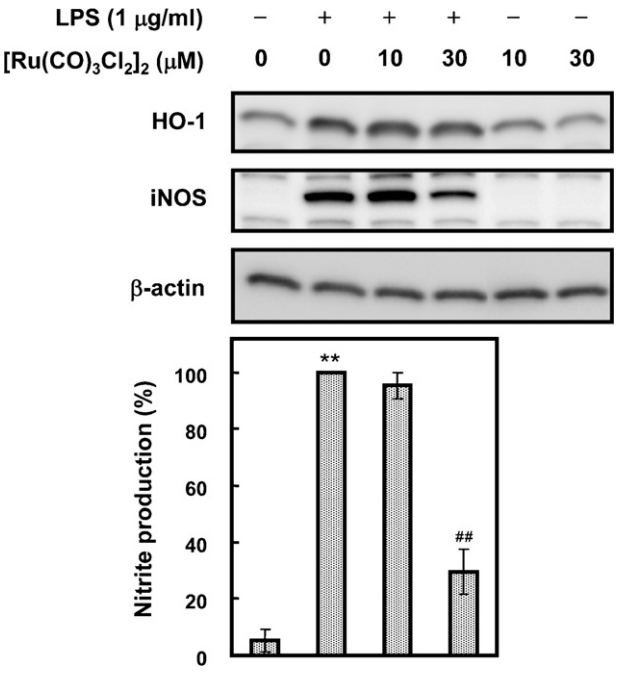

Fig. 7. Effects of treatment with $[Ru(CO)_3Cl_2]_2$ on LPS-stimulated iNOS induction and NO production in BV2 cells. BV2 cells were treated with or without LPS (1 μg/ml) in the absence or presence of $[Ru(CO)_3Cl_2]_2$, a CO donor (10 and 30 μM). Twenty-four hours later, cell lysates were harvested and subjected to Western blot analysis for HO-1, iNOS and β-actin. A representative blot from three independent experiments is shown. Cell culture media were also harvested for measurement of nitrite (mean ± SE, $n = 3$). $^{**}P < 0.01$ vs. cells without compound 1 and without LPS, $^{\#\#}P < 0.01$ vs. cells without compound 1 and with LPS.

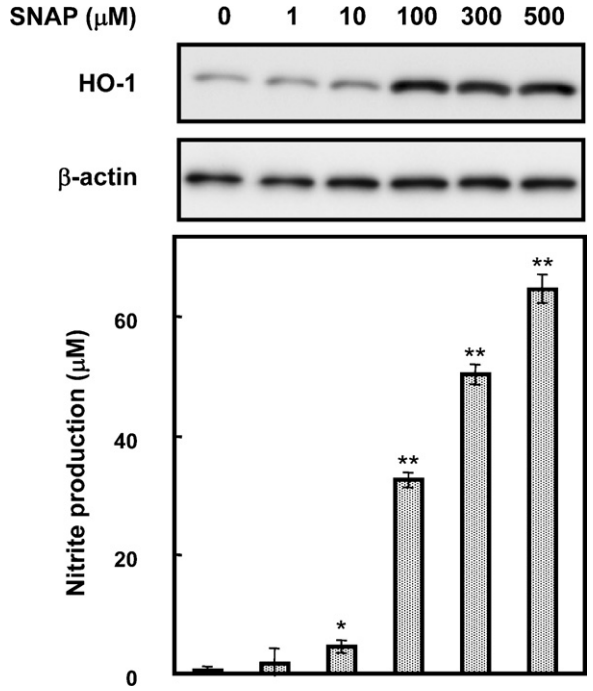

Fig. 8. Effects of treatment with SNAP on HO-1 induction in BV2 cells. BV2 cells were treated with increasing concentrations of SNAP, an NO donor (1, 10, 100, 300 and 500 μM). Six hours later, cell lysates were harvested and subjected to Western blot analysis for HO-1 and β-actin. A representative blot from three independent experiments is shown. Cell culture media were also harvested for measurement of nitrite (mean ± SE, $n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$ vs. cells without SNAP.

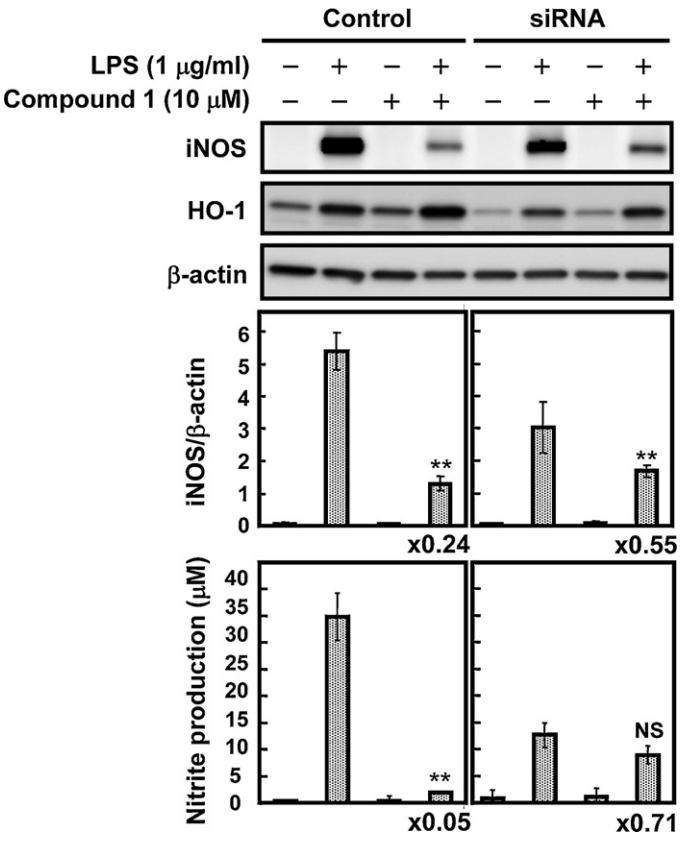

Fig. 9. Effects of HO-1 knockdown on LPS-stimulated iNOS induction and NO production in BV2 cells. BV2 cells were transfected with a control siRNA or HO-1 siRNA. Twenty-four hours after transfection, cells were incubated for 1 h in the absence or presence of 10 μM compound 1, and then treated with or without LPS (1 μg/ml). Twenty-four h after LPS treatment, cell lysates were harvested and subjected to Western blot analysis for HO-1, iNOS and β-actin. A representative blot from three independent experiments is shown along with densitometric data (mean ± SE, $n = 3$). The band intensities were normalized by β-actin. Cell culture media were also harvested for measurement of nitrite. For each siRNA transfection, the fold change relative to cells treated without compound 1 and with LPS is shown (mean ± SE; $n = 3$; $^{**}P < 0.01$; NS, not significant).

involved in the inhibition by compound 1 of LPS-stimulated iNOS induction and NO production.

3.11. Compound 1 inhibits LPS/IFN-γ-stimulated NO production in primary microglial cells

In order to confirm the physiological and pathological relevance of our results obtained in BV2 cultured microglial cells, we studied the effect of compound 1 on NO production in mouse primary microglial cells. We initially treated primary microglial cells with LPS alone, but the LPS-stimulated NO production was relatively small. Therefore, we used IFN-γ (25 ng/ml) as well as LPS (200 ng/ml) to stimulate microglia according to the experimental conditions described in the previous literatures [33–35]. We pretreated cells with compound 1 for 24 h and then incubated with LPS/IFN-γ for 12 h. In support of the observation in BV2 cells (Fig. 1), compound 1 inhibited NO production after LPS/IFN-γ stimulation in primary microglial cells (Fig. 11).

4. Discussion

In the present study, we demonstrated that a chemically synthesized kavalactone derivative, compound 1, inhibits LPS-stimulated iNOS induction and NO production in BV2 microglial cells. At 6 h after LPS challenge, when iNOS induction was not clearly seen, treatment with LPS or compound 1 alone resulted in an increase in HO-1 expression. When treated with both, compound 1 enhanced LPS-stimulated HO-1 induction, which was more evident at 24 h after LPS treatment. In support of these findings, LPS-stimulated activation of Nrf2 signaling and nuclear translocation of Nrf2 were potentiated by compound 1. Treatment with hemin, an inducer of HO-1 expression, and $[Ru(CO)_3Cl_2]_2$, a CO donor, inhibited iNOS induction and NO production in LPS-stimulated BV2 cells. In contrast, knockdown of HO-1 expression by siRNA reduced the inhibitory effects of compound 1 on LPS-stimulated iNOS induction and NO production. Our results indicate that pretreatment with compound 1 potentiates LPS-stimulated Nrf2 activation and HO-1 induction, thereby inhibiting iNOS induction and NO production in BV2 cells (Fig. 12). Furthermore, SNAP, an NO donor, increased HO-1 expression. Based on temporal profile of iNOS and HO-1 expression, we speculated that at an earlier time after LPS treatment, LPS activates Nrf2 signaling and induces HO-1 expression, which in turn inhibits iNOS induction and NO production. At a later time when a significant amount of NO is produced by induction of iNOS caused by LPS stimulation, NO also activates Nrf2 signaling and induces HO-1 expression, further inhibiting iNOS induction and NO production.

We have previously reported that compound 1 ameliorates H_2O_2-induced PC12 cell death through induction of phase II antioxidant enzymes via activation of Nrf2 signaling [22]. The compound 1 increased phosphorylation of ERK1/2 and p38 prior to nuclear translocation of Nrf2, and pretreatment with inhibitors of MEK1/2 and p38 diminished induction of HO-1 caused by compound 1, suggesting that compound 1 may activate Nrf2 signaling by phosphorylation of Nrf2. If compound 1 activated intracellular signaling in BV2 cells, it would be possible that it activates not only Nrf2 signaling but also LPS signaling. Unlike in PC12 cells, treatment with compound 1 did not activate phosphorylation of p38, JNK and Akt, but rather slightly inhibited ERK phosphorylation. This is probably due to cell-type specific differences in the effects of compound 1 on signal transduction. It is therefore unlikely that compound 1 activates Nrf2 signaling by phosphorylation of Nrf2 in BV2 cells.

In addition to Nrf2 phosphorylation, covalent modification by electrophiles of the sulfhydryl groups of Keap1 has been proposed as another mechanism of Nrf2 activation. Naturally derived phytochemicals such as curcumin, caffeic acid phenethyl ester (CAPE) and zerumbone have 2, 1 and 1 α,β-unsaturated carbonyl groups, respectively [5,6,36]. They are supposed to interact with the sulfhydryl groups of Keap1 as electrophiles or Michael reaction acceptors, activating Nrf2 signaling. We have previously reported that sesquiterpene lactones extracted from *Calea urticifolia* (Compositae) and feverfew activate Nrf2 signaling in a manner dependent on the number of α,β-unsaturated carbonyl groups each compound bears [37]. Since compound 1 has one α,β-unsaturated carbonyl group, it is likely that compound 1 directly interacts with Keap1, thereby activating Nrf2 signaling.

It has been reported that a variety of compounds inhibit LPS-stimulated induction of pro-inflammatory genes through suppression of signal transduction. Here, we examined the effects of compound 1 on LPS-stimulated signal transduction. The results showed that compound 1 inhibited LPS-stimulated phosphorylation of ERK, but did not affect that of JNK and Akt. The nuclear translocation of NFκB induced by LPS stimulation was also not inhibited by compound 1. NFκB plays an important role in LPS-stimulated induction of pro-inflammatory genes and the inhibition of NFκB activation is often involved in the reduction of LPS-stimulated gene expression. Our results, however, suggest that the NFκB pathway may not be involved in the inhibition by compound 1 of LPS-stimulated iNOS induction, which is consistent with the finding that compound 1 inhibited induction of iNOS but not COX2 in LPS-stimulated BV2 cells. These results suggest

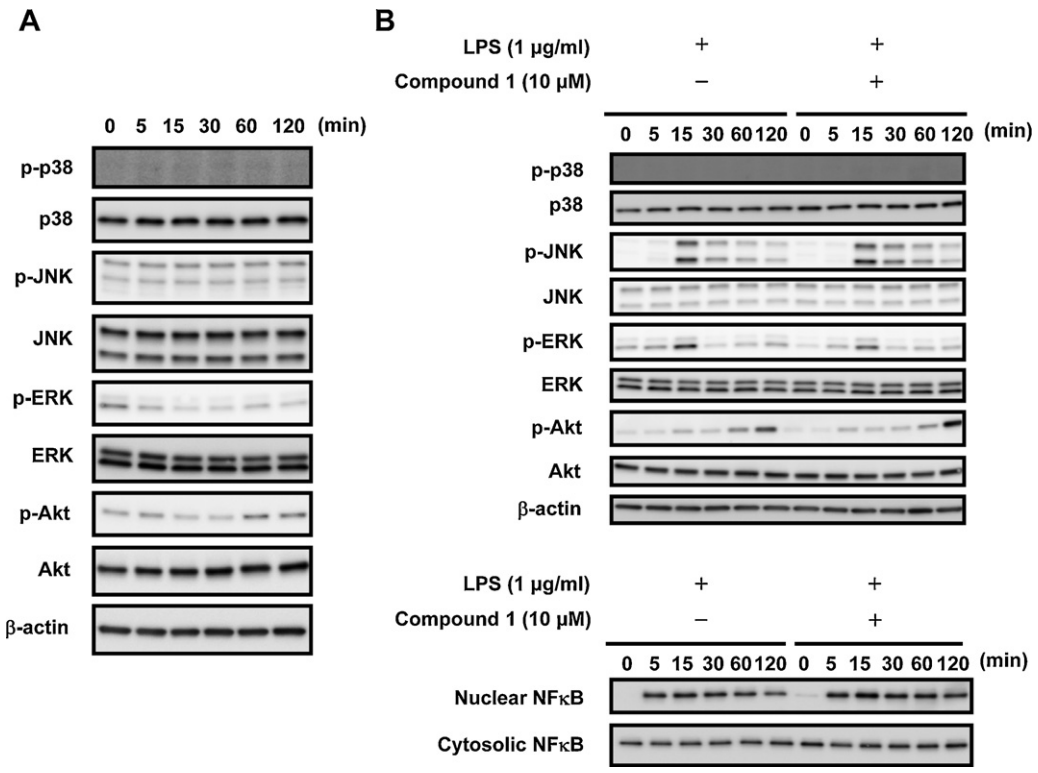

Fig. 10. Effects of compound 1 on signal transduction in BV2 cells. (A) BV2 cells were treated with 10 μM compound 1 alone. At indicated time points, cell lysates were harvested and subjected to Western blot analysis for indicated proteins. A representative blot from three independent experiments is shown. (B) BV2 cells were incubated for 1 h in the absence or presence of 10 μM compound 1, and then treated with LPS (1 μg/ml). At indicated time points after LPS treatment, cell lysates and the cytosolic and nuclear fractions were harvested and subjected to Western blot analysis for indicated proteins. A representative blot from three independent experiments is shown.

that compound 1 suppresses LPS-stimulated iNOS induction and NO production in part through inhibiting ERK phosphorylation in BV2 cells. In addition to transcriptional regulation, iNOS expression has been shown to be also regulated at post-transcriptional and -translational levels [38–42]. For instance, alpha-lipoic acid inhibits the induction of iNOS gene expression at a posttranscriptional step via iNOS mRNA stabilization, rather than promoter activation [38]. Kaempferol inhibits NO production and iNOS protein expression in LPS-stimulated RAW264.7 cells at the translational level [39]. Peroxidized products of EPA/DHA suppress the induction of iNOS gene expression through both of the transcriptional and posttranscriptional steps [40]. The precise mechanisms by which compound 1 inhibits LPS-stimulated iNOS induction through HO-1 induction and CO production remain to be studied.

Naturally derived kavalactones have been shown to pass through the blood brain barrier [43] and ameliorate cerebral

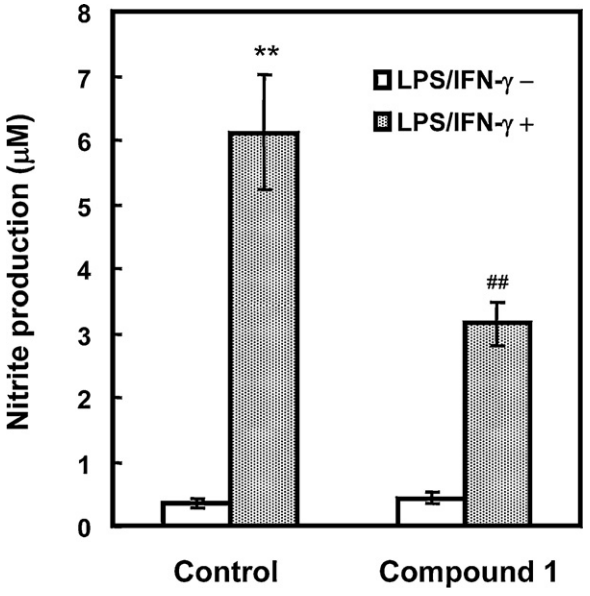

Fig. 11. Effects of compound 1 on LPS/IFN-γ-stimulated NO production in primary cultured microglial cells. Mouse primary cultured microglial cells were incubated for 24 h in the absence (control) or presence of 10 μM compound 1, and then treated with or without LPS (200 ng/ml)/IFN-γ (25 ng/ml). Twelve hours later, cell culture media were harvested for measurement of nitrite (mean ± SE, $n = 3$). $^{**}P < 0.01$ vs. control without LPS/IFN-γ, $^{\#\#}P < 0.01$ vs. control with LPS/IFN-γ.

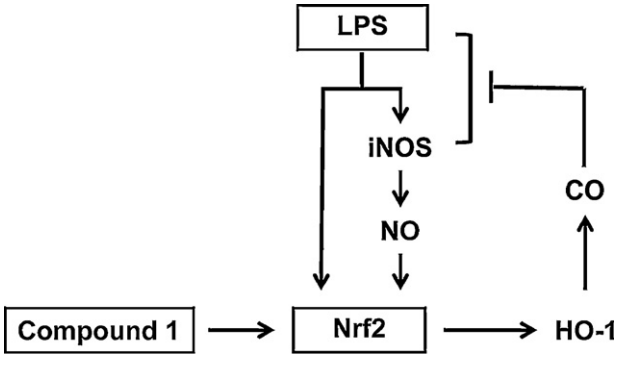

Fig. 12. Schematic representation of molecular mechanisms underlying the inhibitory effect of compound 1 on iNOS induction and NO production. LPS induces iNOS expression and NO production. In parallel, LPS activates Nrf2 signaling and induces HO-1 expression. HO-1 inhibits iNOS induction in part through generation of CO. NO also activates Nrf2 signaling and induces HO-1 expression, inhibiting iNOS induction. After LPS stimulation, HO-1 induction takes place earlier than iNOS induction. The compound 1 enhances LPS-stimulated Nrf2 signaling and HO-1 induction and thereby inhibits iNOS induction and NO production.

infarction in a mouse model [20]. In the present study, we demonstrated that compound 1 as well as naturally derived kavalactones inhibit LPS-stimulated iNOS induction and NO production in BV2 cells. The compound 1 was also found to suppress LPS/IFN-γ-stimulated NO production in primary microglial cells (Fig. 11). On the other hand, we and other group reported that compound 1 and naturally derived kavalactones inhibit oxidative stress-induced PC12 cell death, respectively [21,22]. Taken together, it is suggested that compound 1 as well as naturally derived kavalactones could inhibit oxidative stress in neurons and neuroinflammation in microglia in the brain. It is noteworthy that Nrf2 activation is involved in both processes, but the underlying mechanisms may be different between in neurons and in microglia. Nrf2 may be activated in neurons through activation of signal transduction and covalent modification of the sulfhydryl groups of Keap1 by an α,β-unsaturated carbonyl group, but in microglia mainly through the latter mechanism. Administration of small molecular compounds like compound 1 and naturally derived kavalactones, which are able to reduce both oxidative stress and neuroinflammation through activation of Nrf2 signaling, could be a promising strategy to alleviate neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease.

5. Conclusions

In BV2 microglial cells or mouse primary cultured microglia, chemically synthesized kavalactone derivative, compound 1, enhances LPS-stimulated activation of Nrf2 signaling and HO-1 induction, presumably via covalent modification of Keap1, resulting in inhibition of LPS-stimulated iNOS induction and NO production. The compound 1 has a potential to reduce neuroinflammation in neurodegenerative diseases through activation of Nrf2 signaling.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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