Cytoprotective and anti-inflammatory effects of spinasterol via the induction of heme oxygenase-1 in murine hippocampal and microglial cell lines

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ABSTRACT

Spinasterol, which is isolated from the aerial parts of *Aster scaber* Thunb. (Asteraceae), is involved in various biological activities. In this study, we report the efficacy of spinasterol in effectively modulating the regulation of antioxidative and anti-inflammatory activity through the upregulation of heme oxygenase (HO)-1 in murine hippocampal HT22 cells and BV2 microglia. We showed that spinasterol increased the cellular resistance of HT22 cells to oxidative injury caused by the glutamate-induced cytotoxicity by extracellular signal-regulated kinase (ERK) pathway-dependent expression of HO-1. Furthermore, spinasterol suppressed the lipopolysaccharide (LPS)-induced expression of pro-inflammatory enzymes and inflammatory mediators in BV2 microglia. Spinasterol also suppressed the production of nitric oxide (NO), prostaglandin E2 (PGE$_2$), tumor necrosis factor-α (TNF-α), and interleukin-1β (IL-1β) through extracellular signal-regulated kinase (ERK) pathway-dependent expression of HO-1. These results suggest that spinasterol has a therapeutic potential against neurodegenerative diseases that are caused by oxidative stress and neuroinflammation.

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1. Introduction

A number of diseases that lead to injury of the central nervous system (CNS) are caused by oxidative stress and inflammation in the brain [1]. Oxidative stress or the accumulation of reactive oxygen species (ROS) plays a central role in neuronal injury, cell death, and pathogenesis of neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, and stroke, thereby leading to neuronal death and dysfunction [2–4]. Oxidative glutamate toxicity is a form of nerve cell death, in which glutamate inhibits cystine uptake via the cystine/glutamate antiporter system Xc$^-$, thereby eventually leading to programmed cell death due to glutathione depletion and accumulation of ROS [5,6]. Immortalized mouse hippocampal HT22 cells have been used as in vitro models for studying the mechanism of oxidative glutamate toxicity. This is because neuronal cell death is induced by oxidative damage and not by the activation of ionotropic glutamate receptors, thereby excluding excitotoxicity as a cause of glutamate-induced cell death [7,8].

Several neurodegenerative disorders such as Alzheimer's and Parkinson's diseases are characterized by neuroinflammation of the pathologically affected tissue [9,10]. Microglia, which are brain macrophages, are the primary immune cells of the brain that are activated on injury to the brain, and they release various pro-inflammatory cytokines and inflammatory mediators such as NO, prostaglandins, TNF-α, and IL-1β [11–14]. BV2, an immortalized murine microglia cell lines are widely used as a model of microglia in vitro, because of this cell line retains most of the morphological and functional properties described for primary microglia [15,16]. In previous studies on microglial responses in CNS inflammation, microglial activation was induced by a variety of agents, including the bacterial product LPS, and pro-inflammatory cytokines such as interferon-γ (IFN-γ) and TNF-α [17–19]. Therefore, regulation of oxidative damage and neuroinflammation would be an effective therapeutic approach for alleviating the progression of neurodegenerative diseases.

Heme oxygenase (HO) is an enzyme that catalyzes the degradation of the heme group to produce carbon monoxide (CO), biliverdin, and free iron. HO and its enzymatic by-products appear to play important roles in regulating biological responses, including oxidative stress, inflammation, and cell proliferation [20]. Among the 3 reported HO isoforms (HO-1, -2, -3), HO-1 is highly inducible and expressed in many cell types, including neuronal cells [5,8,21]. The expression of HO-1 also has cytoprotective effects against glutamate-induced oxidative cytotoxicity in HT22 cells [5,22]. Due to its antioxidative effects, HO-1 is considered to be a novel target for the treatment of inflammatory diseases [23–26]. Studies have shown that the anti-inflammatory action of HO-1 is mediated by the inhibition of the production of pro-inflammatory cytokines and chemokines such as TNF-α, IL-1β, IL-6, and MIP-1β in activated macrophages [27,28]. Furthermore, HO-1 and its by-product–carbon monoxide–can suppress the expression of the pro-inflammatory enzymes cyclooxygenase-2 (COX-2) and inducible nitric

oxide synthase (iNOS), thereby decreasing the production of COX-2-derived PGE_2 and iNOS-derived NO [29,30].

Mitogen-activated protein kinase (MAPK) pathway is one of the most common signaling pathways that participate in transducing a variety of extracellular signals to evoke cellular responses. There are 3 members of the MAPK subfamily, namely, extracellular signal-regulated kinases (ERK), p38 kinase, and c-Jun N-terminal kinase (JNK) [31–33]. MAPK activation leads to the phosphorylation of several transcription factors and is responsible for the regulation of genes involved in the control of fundamental cellular processes such as proliferation, stress responses, apoptosis, and immune defense [34]. MAPK activation modulates the expression of several genes and proteins, including HO-1 [35].

Spinasterol, which is a biologically active compound isolated from the aerial parts of *Aster scaber* Thunb. (Asteraceae), exhibits various pharmacological activities, including anti-tumor, antiulcerogenic, and anti-carcinogenic activities [36–38]. Several published studies have shown that spinasterol also has anti-inflammatory effects [39]. However, the mechanisms underlying the anti-inflammatory effect of spinasterol remain to be elucidated. As a part of our ongoing research to identify phytochemicals isolated from natural sources that can induce HO-1 expression *in vitro* [8,40], spinasterol was shown to induce significant expression of HO-1 in mouse hippocampal HT22 cells and BV2 microglia cells. In this study, we showed that spinasterol increased cellular resistance of HT22 to oxidative injury caused by glutamate-induced cytotoxicity by the ERK pathway-dependent expression of HO-1. Using BV2 microglia as the model, we also investigated the possible involvement of HO-1 in the anti-inflammatory activity of spinasterol, and examined whether spinasterol-mediated HO-1 expression correlates with the inhibition of LPS-induced pro-inflammatory mediators such as NO, PGE_2, TNF-α, and IL-1β. Further, we provided evidence to support the view that HO-1 plays an important role in mediating the antioxidative and anti-inflammatory effects of spinasterol.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and other tissue culture reagents were purchased from Gibco BRL Co. (Grand Island, NY, USA). Tin protoporphyrin IX (SnPP IX; inhibitor of HO activity) was obtained from Porphyrin Products (Logan, UT, USA). Cobalt protoporphyrin IX (CoPP; HO-1 inducer) and Trolox were obtained from Sigma Chemical Co. (St. Louis, MO, USA). PD98059, SP600125, and SB203580 were obtained from Calbiochem (Darmstadt, Germany). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Spinasterol (Fig. 1) was isolated from *A. scaber* Thunb. as described previously [41]. Primary antibodies, including mouse/goat/rabbit anti-HO-1, COX-2, iNOS, and secondary antibodies were purchased from Santa Cruz Biotechnology (Heidelberg, Germany). Enzyme-linked immunosorbent assay (ELISA) kits for PGE_2, TNF-α, and IL-1β were purchased from R & D Systems, Inc. (Minneapolis, MN, USA).

2.2. Cell culture and viability assay

Mouse hippocampal HT22 cells were received from Dr In hee Mook at Seoul National University (Seoul, Korea), and the BV2 microglia cells were received from Prof. Hyun Park at Wonkwang University (Iksan, Korea). The cells were maintained at 5×10^5 cells/ml in DMEM supplemented with 10% heat-inactivated FBS, penicillin G (100 U/ml), streptomycin (100 mg/l), and L-glutamine (2 mM) and were incubated at 37 °C in a humidified atmosphere containing 5% CO_2 and 95% air. For determination of cell viability, 50 mg/ml of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was added to 1 ml of cell suspension (1×10^5 cells per 1 ml in 96-well plates) and incubated for 4 h. The formazan formed was dissolved in acidic 2-propanol, and optical density was measured at 590 nm.

2.3. Reactive oxygen species measurement

For measurement of ROS, HT22 cells (2.5×10^4 cells/ml in 24-well plates) were treated with 5 mM glutamate in the presence or absence of spinasterol or SnPP (HO inhibitor) and incubated for 8 h. After washing with PBS, the cells were stained with 10 μM 2′,7′-dichloro-fluorescein diacetate (DCFDA) in Hanks' balanced salt solution for 30 min in the dark. The cells were then washed twice with PBS and extracted using 1% Triton X-100 in PBS for 10 min at 37 °C. Fluorescence was recorded at an excitation wavelength of 490 nm and emission wavelength of 525 nm (Spectramax Gemini XS; Molecular Devices, Sunnyvale, CA).

2.4. Nitrite assay

The nitrite concentration in the medium was measured as an indicator of NO production as per the Griess reaction. Each supernatant (100 μl) was mixed with the same volume of the Griess reagent (Solution A: 222488; Solution B: S438081, Sigma); absorbance of the mixture at 525 nm was determined using an ELISA plate reader.

2.5. PGE_2 assay

BV2 microglia were cultured in 24-well plates, pre-incubated for 12 h with different concentrations of spinasterol, and then stimulated for 18 h with LPS. The culture supernatant (100 μl) was collected to determine the PGE_2 concentration using the ELISA kit (R & D Systems, Minneapolis, MN, USA).

2.6. TNF-α and IL-1β assay

BV2 microglia were cultured in 24-well plates, pre-incubated for 12 h with various concentrations of spinasterol, and then stimulated for 18 h with LPS. Culture supernatants were collected, and the concentration of TNF-α and IL-1β was determined using ELISA kits (R & D Systems) as per the manufacturer's instructions.

2.7. Western blot analysis

HT22 and BV2 cells were harvested and pelleted by centrifugation at $200 \times g$ for 3 min. Then, the cells were washed with phosphate-buffered saline (PBS) and lysed with 20 mM Tris–HCl buffer (pH 7.4) containing a protease inhibitor mixture (0.1 mM phenylmethanesulfonyl fluoride, 5 mg/ml aprotinin, 5 mg/ml pepstatin A, and 1 mg/ml chymostatin).
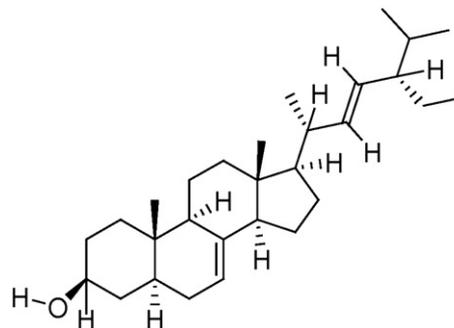

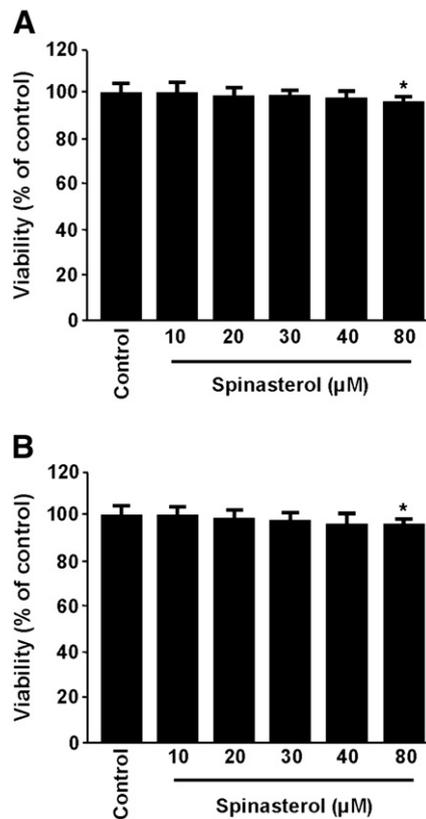**Fig. 1.** Chemical structure of spinasterol.

Fig. 2. Effects of spinasterol on cell viability. HT22 cells (A) and BV2 microglia (B) were incubated for 12 h with various concentrations of spinasterol (10–80 μM). Cell viability was determined as described in Materials and methods. Bar represents the mean ± SD of 3 independent experiments. *P<0.05 vs. control.

Protein concentration was determined using the Lowry protein assay kit (P5626; Sigma). An equal amount of protein from each sample was resolved using 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then electrophoretically transferred onto a Hybond enhanced chemiluminescence (ECL) nitrocellulose membrane (Bio-Rad, Hercules, CA). The membrane was blocked with 5% skimmed milk and sequentially incubated with the primary antibody (Santa Cruz Biotechnology, CA) and the horseradish peroxidase-conjugated secondary antibody followed by ECL detection (Amersham Pharmacia Biotech, Piscataway, NJ).

2.8. HO activity

To determine HO activity, we followed the method described by Motterlini et al. [42]. Briefly, after the incubation process, the cells were washed twice with PBS, gently scraped off the dish, and centrifuged ($1000 \times g$ for 10 min at 4 °C). The cell pellet was suspended in $MgCl_2$ (2 mM) phosphate (100 mM) buffer (pH 7.4), frozen at −70 °C, thawed 3 times, and finally sonicated on ice before centrifugation at $18,000 \times g$ for 10 min at 4 °C. The supernatant (400 μl) was added to an NADPH-generating system containing 0.8 mM NADPH, 2 mM glucose-6-phosphate, 0.2 units of glucose-6-phosphate-L-dehydrogenase, and 2 mg protein of rat liver cytosol prepared from the $15,000 \times g$ supernatant fraction as a source of biliverdin reductase, potassium phosphate buffer (100 for 1 h at mM, pH 7.4), and hemin (10 μM) in a final volume of 200 μl. The reaction was performed 37 °C in the dark and terminated by the addition of 1 ml of chloroform. The amount of extracted bilirubin was calculated by determining the difference in absorption at 464 and 530 nm using a quartz cuvette (extinction coefficient, 40 mM^{-1} cm^{-1} for bilirubin).

2.9. Statistical analysis

Data were expressed in terms of mean ± SD of at least 3 independent experiments. To compare 3 or more groups, one-way analysis of variance (ANOVA) was used followed by the Newman–Keuls post hoc test. Statistical analysis was performed using GraphPad Prism software version 3.03 (GraphPad Software Inc, San Diego, CA).

3. Results

3.1. Effects of spinasterol on cell viability

To determine the cytotoxic potential of spinasterol, we evaluated its effect on the viability of HT22 cells (Fig. 2A) and BV2 microglia (Fig. 2B). MTT assay performed at 40 μM of spinasterol, revealed no cytotoxic effects both in HT22 cells and BV2 microglia.

3.2. Effects of spinasterol on glutamate-induced cytotoxicity and inhibition of ROS generation in HT22 cells

Treatment with glutamate for 12 h increased HT22 cell death by 62% as compared to the untreated cells, and at non-cytotoxic concentrations, spinasterol (10, 20, 30, and 40 μM) increased viability dose-dependently (Fig. 3A). Glutamate also doubled ROS production, and spinasterol effectively suppressed this induction (Fig. 3B). Trolox, which is a well-known antioxidative agent, was used as a positive
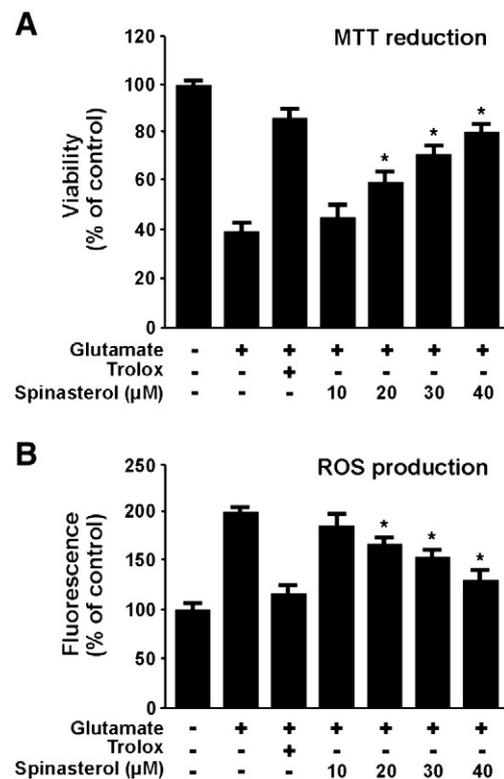

Fig. 3. Effects of spinasterol on glutamate-induced oxidative neurotoxicity (A), and inhibition of ROS generation (B) in HT22 cells. (A) Cells were pretreated for 12 h with indicated concentrations of spinasterol and then incubated for 12 h with glutamate (5 mM). (B) Exposure of HT22 cells to 5 mM glutamate for 12 h increased ROS production. Spinasterol effectively inhibited glutamate-mediated ROS production. Data represent mean values of 3 experiments ± SD. *P<0.05 compared to the group treated with glutamate. Trolox (50 μM) was used as a positive control.

control, and it showed a significant cytoprotective effect and ROS-scavenging activity at a concentration of 50 μM.

3.3. Effects of spinasterol on LPS-induced pro-inflammatory enzymes and pro-inflammatory cytokines in BV2 microglia

The levels of iNOS and COX-2 expression were measured in BV2 microglia challenged with LPS (0.5 μg/ml) in the presence or absence of spinasterol at the non-cytotoxic concentrations (10–40 μM). Pretreatment of the BV2 microglia with spinasterol for 12 h resulted in suppressed iNOS expression (Fig. 4A) and iNOS-derived NO (Fig. 4C). Under the same conditions, spinasterol also decreased COX-2 expression (Fig. 4B), and was also found to reduce the COX-2-derived PGE_2 production (Fig. 4D). Moreover, the effects of spinasterol on the LPS-induced TNF-α and IL-1β production were examined by an enzyme immunoassay in which BV2 microglia were pre-incubated with spinasterol for 12 h followed by LPS stimulation. As shown in Fig. 4E and F, spinasterol also decreased TNF-α and IL-1β production in a concentration-dependent manner.

3.4. Effects of spinasterol on HO-1 expression and HO activity

We examined the effects of spinasterol on HO-1 protein expression at non-cytotoxic concentrations (10–40 μM) by treating the HT22 cells and BV2 microglia with this agent for 12 h. Spinasterol dose-dependently increased HO-1 expression in HT22 cells (Fig. 5A) and BV2 microglia (Fig. 5C). At a concentration of 40 μM, HO-1 expression was first detected at 6 h, increased to a maximum at around 18 h, and reduced after 24 h in HT22 cells (Fig. 5B) and BV2 microglia (Fig. 5D).

CoPP (20 μmol·L^{-1}), a well-known HO-1 inducer, was used as the positive control. In accordance with the concentration-dependent HO-1 expression, spinasterol also increased HO activity (Fig. 5E and F). This enhanced HO activity has directly correlated with HO-1 protein level, because of the SnPP, an HO activity inhibitor, decreased spinastero induced HO activity (Fig. 6).

3.5. Effects of HO-1 expression on glutamate-induced oxidative neurotoxicity and inhibition of the pro-inflammatory mediators by spinasterol

In recent studies, HO-1 exerted cytoprotective effects against glutamate-induced oxidative cytotoxicity in HT22 cells [2,8]. Thus, we examined whether spinasterol-induced HO-1 expression mediated these cytoprotective effects. HT22 cells were co-treated with 40 μM of spinasterol for 12 h in the absence or presence of SnPP, which is an inhibitor of HO-1 activity. SnPP significantly inhibited the spinasterol-mediated cytoprotection (Fig. 5A). The spinasterol-induced HO-1 expression was also required for suppressing glutamate-induced ROS generation (Fig. 5B). HO-1 has been shown to downregulate inflammation in microglia [22]. Because pre-incubation of the BV2 microglia with spinasterol markedly inhibited LPS-induced pro-inflammatory enzymes and pro-inflammatory cytokines (Fig. 3), and because spinasterol was able to induce HO-1 expression (Fig. 4) in BV2 microglia, we examined whether spinasterol-mediated HO-1 induction could be responsible for the inhibition of iNOS-derived NO, COX-2-derived PGE_2, TNF-α, and IL-1β production. BV2 microglia were pretreated with spinasterol for 12 h in the presence of SnPP, followed by LPS stimulation. As shown in Fig. 5, SnPP treatment
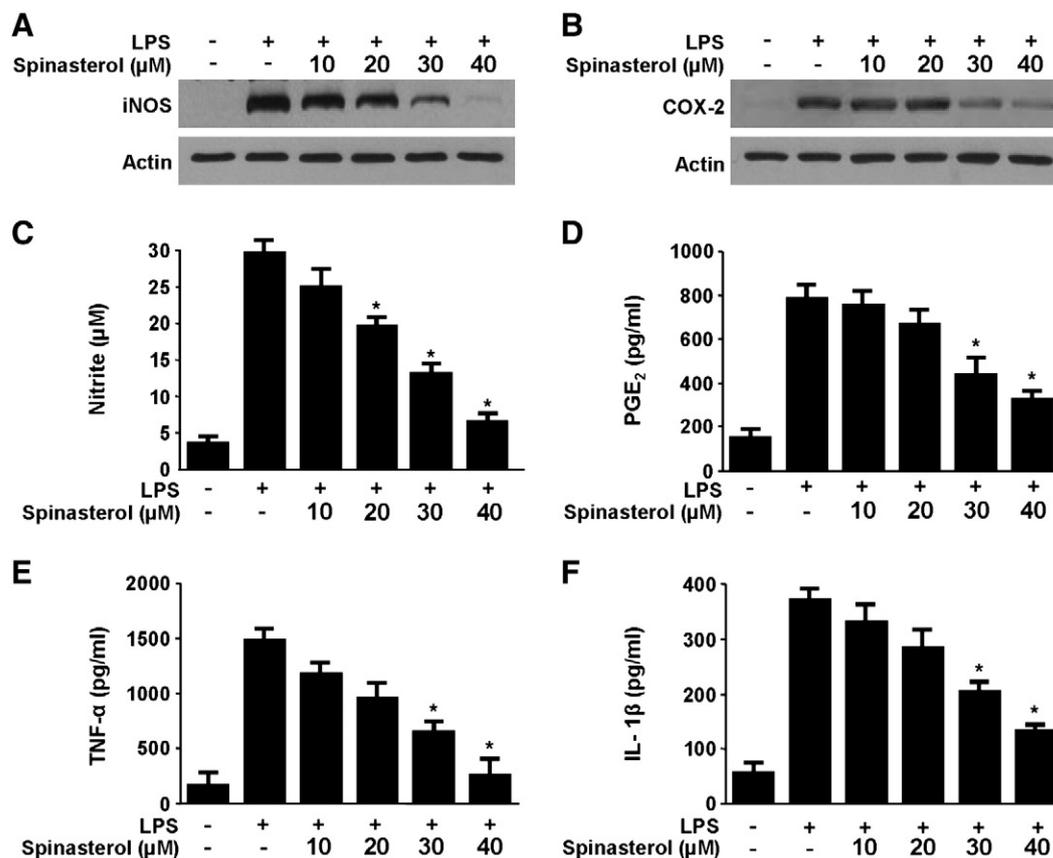

Fig. 4. Effects of spinasterol on LPS-induced inducible iNOS and COX-2 protein expression and NO, PGE_2, and pro-inflammatory cytokine levels in BV2 microglia. BV2 microglia were pretreated for 12 h with indicated concentrations of spinasterol and stimulated for 18 h (A, C, D, E, F) or 6 h (B) with LPS (0.5 μg/ml). Western blot analyses for iNOS and COX-2 expression (A, B) were performed as described in Materials and methods. Representative blots of 3 independent experiments are shown. The concentrations of NO, PGE_2, tumor necrosis TNF-α, and IL-1β (C, D, E, and F) were determined as described under Materials and methods. Data represent mean values of 3 experiments ± SD. *$P<0.05$ compared to the group treated with LPS.
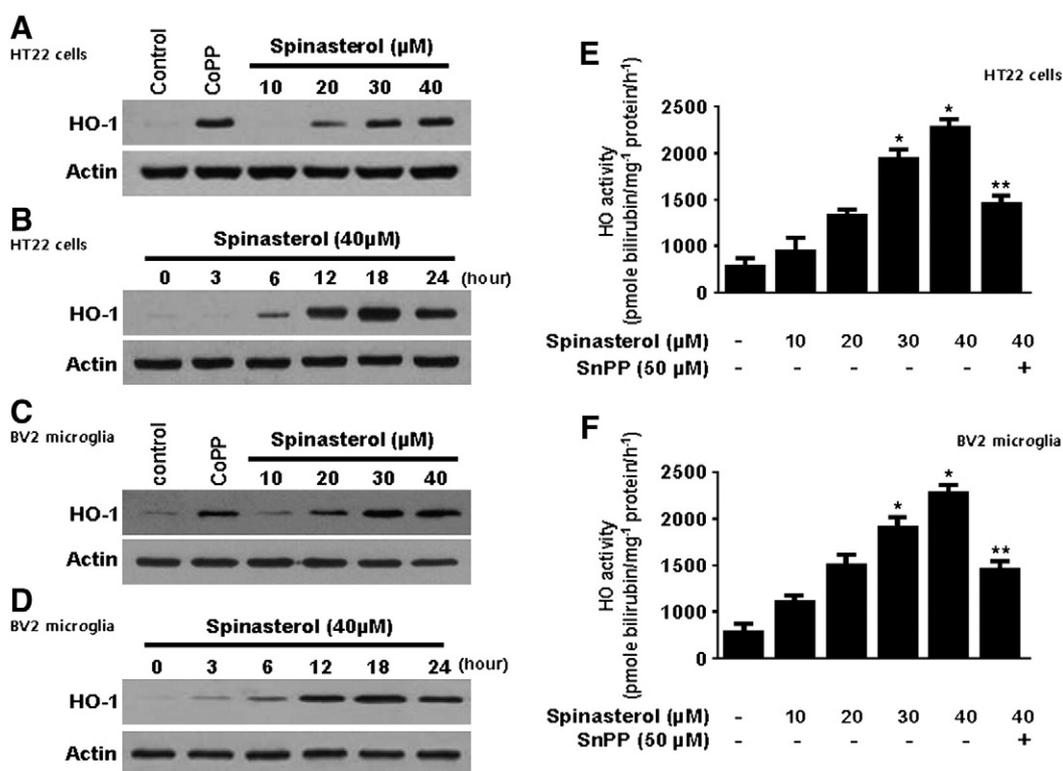
Fig. 5. Effects of spinasterol on HO-1 expression and HO activity in HT22 (A, B, E) and BV2 microglia (C, D, F). HT22 cells and BV2 microglia were incubated for 12 h with indicated concentrations of spinasterol (A, C) and periods with 40 μM of spinasterol (B, D). The HO-1 inducer CoPP, increased HO-1 expression at 20 μM (A,C). Western blot analysis for HO-1 expression was performed as described in Materials and methods, and representative blots of 3 independent experiments are shown. HO activity was determined via bilirubin formation at 12 h after treatment with various concentrations of spinasterol or SnPP (HO inhibitor) in HT22 cells and BV2 microglia (E, F). HO activity was determined as described in Materials and methods. Data represent the mean values of 3 experiments ± SD. *$P<0.05$ compared to the control group; **$P<0.05$ compared to the group treated with spinasterol.

partially reversed the inhibitory effects of spinasterol on NO, PGE$_2$, TNF-α, and IL-1β production (Fig. 5C–F).

3.6. Involvement of the MAPK pathways in spinasterol-induced HO-1 expression

Several studies have reported that the activation of the MAPK pathways contributed to the induction of HO-1 [35]. Therefore, we examined the effect of spinasterol on the activation of MAPKs in HT22 cells and BV2 microglia. At a concentration of 40 μM, which strongly induced HO-1, spinasterol activated the ERK pathway and increased ERK phosphorylation both in HT22 cells and BV2 microglia. Phosphorylation of ERK was observed 15 min after spinasterol treatment, and was sustained up to 60 min after spinasterol treatment (Figs. 7A, 8A). In contrast, phosphorylation of JNK and p38 kinases was not seen at any time. Furthermore, to investigate the role of MAPK in HO-1 expression in HT22 cells, we examined the effects of specific inhibitors of ERK (PD98059), JNK (SP600125), and p38 (SB203580) on HO-1 levels by western blot analysis. We found that spinasterol-induced HO-1 expression was inhibited by the ERK pathway inhibitor, whereas the JNK and p38 inhibitors had no effect (Fig. 7B). As expected, treatment with the ERK inhibitor abolished spinasterol-induced cytoprotection, but inhibitors of the JNK or p38 MAPK pathways did not (Fig. 7C). The ERK inhibitor did not exert cytotoxic effects our experimental conditions (data not shown).

In BV2 microglia, at a concentration of 40 μM, spinasterol only activated the ERK pathway and increased ERK phosphorylation and not p38 or JNK. (Fig. 8A). Furthermore, we found that the ERK pathway inhibitor significantly reduced spinasterol-induced HO-1 expression, whereas the JNK and p38 inhibitors did not (Fig. 8B). The ERK inhibitor

was not cytotoxic under our experimental conditions (data not shown).

4. Discussion

Oxidative stress and neuroinflammation have been implicated in many neurodegenerative diseases [43]. Therefore, naturally occurring compounds having both antioxidative and anti-inflammatory effects may offer a promising strategy for therapeutic application. Previous studies have demonstrated that several natural products such as macelignan and xanthorrhizol have antioxidant and anti-inflammatory activities in hippocampal and microglia cells [44,45]. HO-1–an enzyme essential for heme degradation–has been shown to exert antioxidative and anti-inflammatory effects under various conditions. HO-1 system also appeared to be an important therapeutic target in neuronal oxidative damage and inflammation [5,22,46]. As a part of our ongoing research to identify phytochemicals isolated from natural sources that can induce HO-1 in vitro [8,40], spinasterol was shown to significantly induce HO-1 expression in HT22 cells and BV2 microglia.

Glutamate toxicity is a major contributor to pathological cell death within the nervous system. In this study, we examined the protective effects of spinasterol against glutamate-induced cytotoxicity in HT22 cells. At the non-cytotoxic concentrations (10–40 μM), spinasterol inhibited glutamate-induced cell death in a dose-dependent manner 12 h after treatment, and also effectively suppressed glutamate-induced ROS generation. In our previous studies, we have demonstrated that HO-1 expression appeared to play key roles in cytoprotection in HT22 cells [40]. We have provided evidence for the induction of HO-1 expression by spinasterol in HT22 cells and shown that spinasterol-induced HO-1 protein expression and HO activity occurred in a
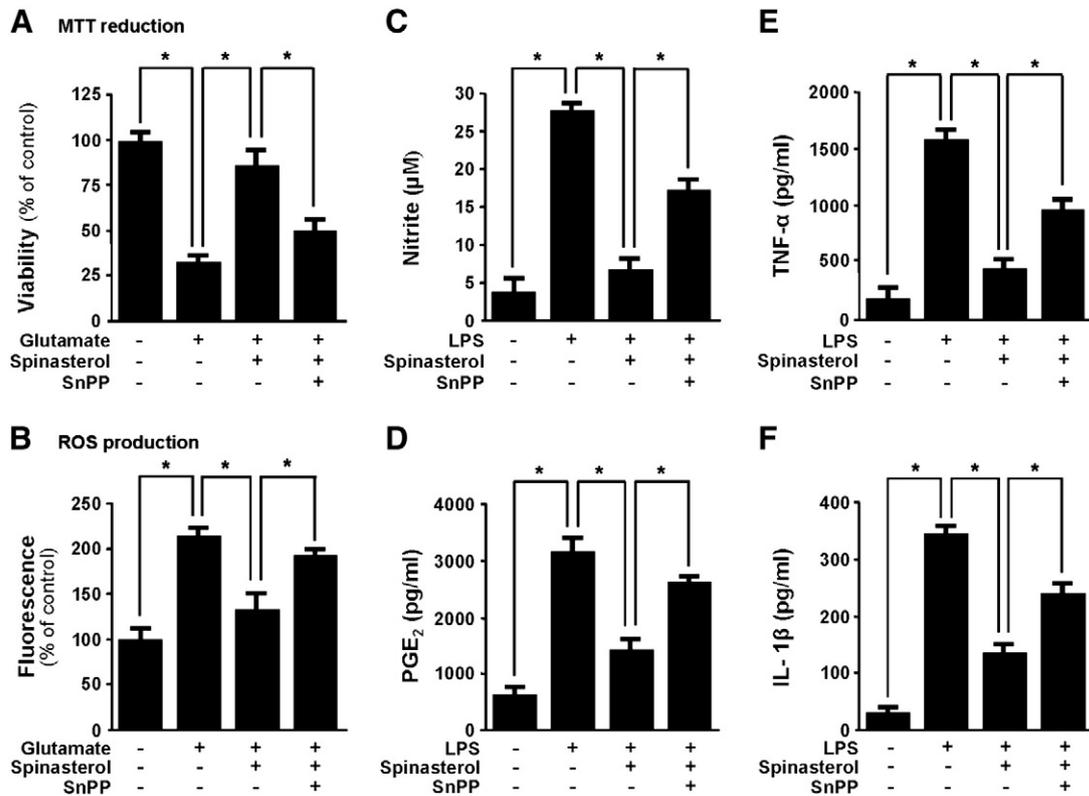
Fig. 6. HO-1 mediates the suppressive effect of spinasterol on glutamate-induced oxidative neurotoxicity, and LPS-stimulated pro-inflammatory mediator production. HT22 cells (A, B) were pretreated for 6 h with spinasterol (40 μM) in the presence or absence of SnPP (50 μM), and then incubated for 12 h with glutamate (5 mM). BV2 cells (C, D, E, F) were pretreated for 3 h with spinasterol (40 μM) in the presence or absence of SnPP (50 μM), and stimulated for 24 h with LPS (0.5 μg/ml). The concentrations of NO, PGE₂, TNF-α, and IL-1β were determined as described in Materials and methods. SnPP was pretreated with HT22 cells and BV2 microglia for 3 h in this experiment. Data represent mean values of 3 experiments ± SD. *P<0.05.
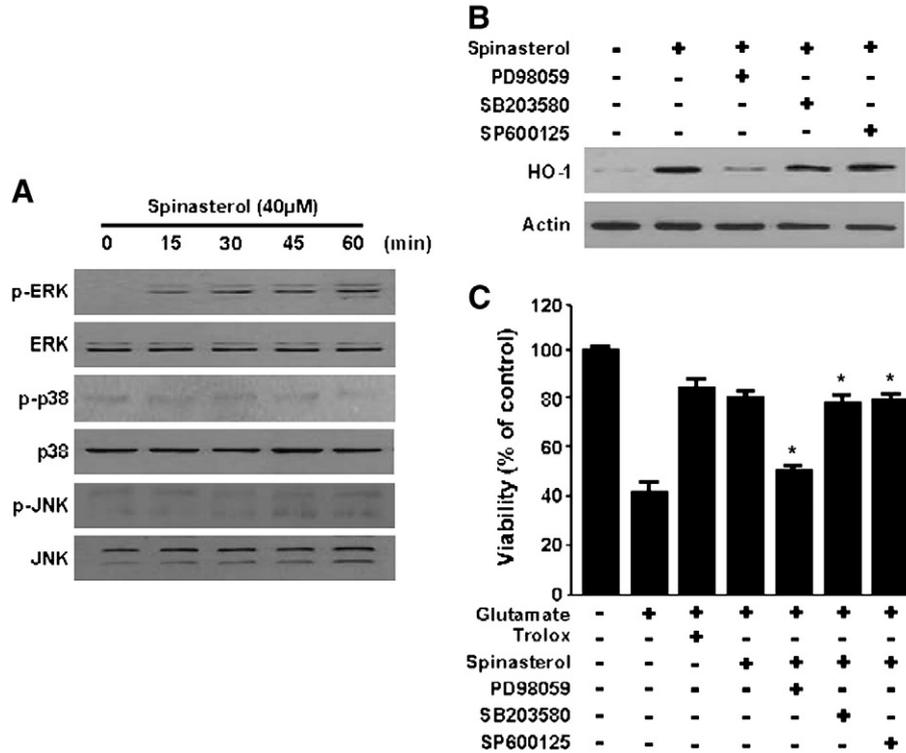

Fig. 7. Effects of spinasterol-induced MAPK activation on HO-1 expression and glutamate-induced neurotoxicity in HT22 cells. (A) Cells were treated with 40 μM of spinasterol for the indicated times. Cell extracts were analyzed by western blot with antibodies specific for phosphorylated extracellular signal-regulated kinase (p-ERK), phosphorylated JNK (p-JNK), or phosphorylated p38 (p-p38). Membranes were stripped and re-probed for total form of each MAPK antibody as a control, and the representative blots of 3 independent experiments are shown. (B) Cells were pretreated for 1 h with the specific inhibitors, PD98059 (40 μM), SP600125 (25 μM), and SB203580 (20 μM), and then treated with spinasterol (40 μM) for 12 h. Western blot analyses for HO-1 expression were performed as described in Materials and methods, and representative blots of 3 independent experiments are shown. (C) Cells untreated or treated with spinasterol (40 μM) in the presence or absence of each specific inhibitor for 12 h were exposed to 5 mM glutamate for 8 h. Each bar represents the mean ± SD of 3 independent experiments. *P<0.05 compared to the group treated with glutamate. Trolox (50 μM) was used as a positive control.
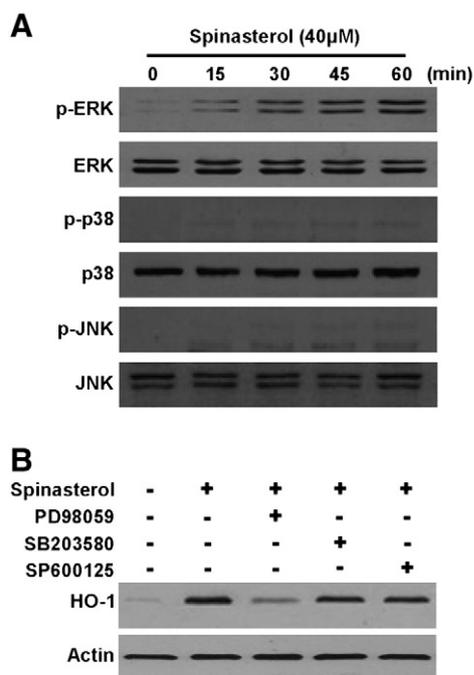
Fig. 8. Effects of spinasterol on MAPK activation, and MAPK-dependent HO-1 expression in BV2 microglia. (A) Cells were treated with 40 µM spinasterol for the indicated times. Cell extracts were analyzed by western blot with antibodies specific for p-ERK, p-JNK, or p-p38. Membranes were stripped and re-probed for total form of each MAPK antibody as a control, and the representative blots of 3 independent experiments are shown. (B) Cells were pretreated for 1 h with the specific inhibitors PD98059 (40 µM), SP600125 (25 µM), and SB203580 (20 µM), and then treated with spinasterol (40 µM) for 12 h. Western blot analyses for HO-1 expression were performed as described in Materials and methods, and representative blots of 3 independent experiments are shown.

concentration- and time-dependent manner. Furthermore, the involvement of HO-1 in the antioxidant activity of spinasterol was also confirmed using a specific HO competitive inhibitor–SnPP–which significantly blocked spinasterol-mediated inhibition of glutamate-induced cell death. The induction of HO-1 expression was also required to suppress glutamate-induced ROS generation. The findings of this study strongly indicate that the observed cytoprotective effects of spinasterol might be due to HO-1 expression.

Over-activation of microglia and increased production of inflammatory mediators may exacerbate neuronal damage [12,47,48]. Upon activation, microglia also promoted neuronal injury through the release of various pro-inflammatory cytokines and inflammatory mediators, such as NO, prostaglandins, TNF-α, and IL-1β [12–14]. Pretreatment with 10–40 µM of spinasterol suppressed iNOS and COX-2 expression in LPS-stimulated BV2 microglia, thereby inhibiting iNOS-derived NO as well as COX-2-derived PGE₂ production. In addition, spinasterol also inhibited LPS-induced TNF-α and IL-1β production. This finding suggests that spinasterol, at least in LPS-stimulated microglia, exerts its anti-inflammatory effects by limiting the expression of the pro-inflammatory enzymes and inhibiting the secretion of the pro-inflammatory cytokines. Furthermore, spinasterol also increased HO-1 expression and HO activity dose-dependently. Several reports indicated that upregulation of HO-1 has been responsible for the anti-inflammatory action in microglia [24]. The present study further examined whether spinasterol inhibited LPS-induced pro-inflammatory mediators, such as iNOS-derived NO, COX-2-derived PGE₂, TNF-α, and IL-1β by increasing the expression of HO-1. Our results indicate that the inhibition of HO activity by the HO inhibitor, SnPP, had partially reversed the inhibitory effects of spinasterol on PGE₂, NO, TNF-α, and IL-1β production in LPS-stimulated BV2 microglia.

MAPK is one of the most common signaling pathways that participate in transducing a variety of extracellular signals to evoke cellular responses. The MAPK activation also modulates the expression

of several genes and proteins, including the activation of HO-1 [33,35]. In this study, activation of the MAPK pathway appeared to be involved in spinasterol-induced HO-1 expression in HT22 cells and BV2 microglia. At a concentration of 40 µM, which strongly induced the levels of HO-1, spinasterol activated the ERK pathway and increased ERK phosphorylation in HT22 cells. Furthermore, treatment of cells with specific protein kinase inhibitors demonstrated that the ERK pathway played a crucial role in the induction of HO-1. As expected, treatment with the ERK inhibitor abolished spinasterol-induced cytoprotection. In addition, spinasterol also had effects on MAPK activation in BV2 microglia. At a concentration of 40 µM, spinasterol activated the ERK pathway and increased ERK phosphorylation in BV2 microglia. Based on these results, we suggest that spinasterol-induced HO-1 expression is directly related to the ERK pathway because the ERK inhibitor, PD98059, influenced the spinasterol-induced change in the HO-1 protein levels, while the inhibitors of JNK and p38 pathways did not display any significant influence (Fig. 9).

HO-1 induction by low-molecular weight compounds might play an important role in the neuroprotection of CNS neurons [22]. Since spinasterol, as confirmed in this study, can actively induce the expression of the HO-1 in mouse hippocampal HT22 cells and BV2 microglia cells, possibility cannot be ruled out that the antioxidative and anti-inflammatory effects of spinasterol are mediated, partially, by the products of HO-1 enzyme reaction, namely, CO, bilirubin, and/or biliverdin.

In conclusion, spinasterol showed potent cytoprotective effects against glutamate-induced neurotoxicity in mouse hippocampal HT22 cells, possibly, through the ERK pathway-dependent expression of HO-1. We investigated the anti-inflammatory activity of spinasterol in microglial activation by LPS. We showed that spinasterol suppresses pro-inflammatory mediators through the ERK pathway-dependent expression of anti-inflammatory HO-1 in BV-2 microglia. The results of our study demonstrate the importance of HO-1 in mediating the antioxidative and anti-inflammatory effects of spinasterol in mouse hippocampal and microglial cells and reveal the possible therapeutic value of spinasterol for the prevention of neurodegenerative disease progression.
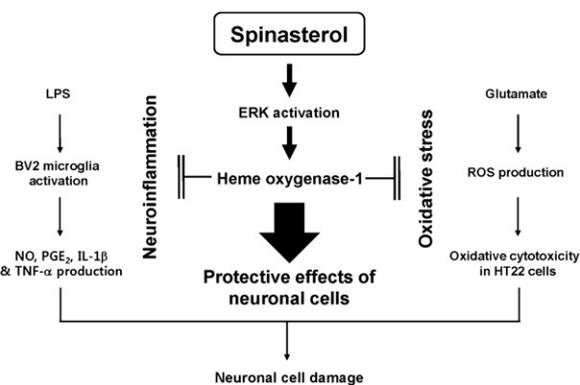
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Fig. 9. The pathway scheme of the anti-inflammatory and cytoprotective effects of spinasterol in BV2 microglia and HT22 cells. Spinasterol protects HT22 cells from glutamate-induced oxidative cytotoxicity, via the ERK pathway-dependent HO-1 expression. Furthermore, spinasterol suppressed the LPS-induced expression of pro-inflammatory enzymes and inflammatory mediators via the ERK pathway-dependent HO-1 expression in BV2 microglia. This study indicates that spinasterol effectively modulates the regulation of antioxidative and anti-inflammatory action, via the ERK pathway-dependent HO-1 expression in BV2 microglia and HT22 cells.

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