Anti-Inflammatory Mechanism of Compound K in Activated Microglia and Its Neuroprotective Effect on Experimental Stroke in Mice

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ABSTRACT

Microglial activation plays a pivotal role in the pathogenesis of various neurologic disorders, such as cerebral ischemia, Alzheimer’s disease, and Parkinson’s disease. Thus, controlling microglial activation is a promising therapeutic strategy for such brain diseases. In the present study, we found that a ginseng saponin metabolite, compound K [20-O-D-glucopyranosyl-20(S)-protopanaxadiol], inhibited the expressions of inducible nitric-oxide synthase, proinflammatory cytokines, monocyte chemotactic protein-1, matrix metalloproteinase-3, and matrix metalloproteinase-9 in lipopolysaccharide (LPS)-stimulated BV2 microglial cells and primary cultured microglia. Subsequent mechanistic studies revealed that compound K suppressed microglial activation via inhibiting reactive oxygen species, mitogen-activated protein kinases, and nuclear factor-κB/activator protein-1 activities with enhancement of heme oxygenase-1/antioxidant response element signaling. To address the anti-inflammatory effects of compound K in vivo, we used two brain disease models of mice: sepsis (systemic inflammation) and cerebral ischemia. Compound K reduced the number of Iba1-positive activated microglia and inhibited the expressions of tumor necrosis factor-α and interleukin-1β in the LPS-induced sepsis brain. Furthermore, compound K reduced the infarct volume of ischemic brain induced by middle cerebral artery occlusion and suppressed microglial activation in the ischemic cortex. The results collectively suggest that compound K is a promising agent for prevention and/or treatment of cerebral ischemia and other neuroinflammatory disorders.

Introduction

Microglia are major immune cells in the central nervous system, which are readily activated after brain injury or during neurodegenerative processes and secrete growth factors, proinflammatory/anti-inflammatory cytokines, reactive oxygen species (ROS), nitric oxide (NO), and glutamate (Block and Hong, 2005; Stolp and Dziegielewska, 2009). Although microglial activation is necessary and important for host defense, overactivation of microglia is neurotoxic. Microglia are also activated after ischemic stroke and produce cytokines, triggering neuronal death in response to ischemic injury (Wang et al., 2007). Within 3 days of a stroke, various inflammatory molecules are concomitantly up-regulated in the brain, cerebrospinal fluid, and blood, thus continuous brain loss is expected during that time. If inflammation can be suppressed, progressive brain loss after a stroke may be prevented and the clinical outcome improved (Wang et al., 2007). Thus, the development of agents that reduce microglial activation and their proinflammatory responses is considered to be an important therapeutic strategy for neuroinflammatory disorders such as cerebral ischemia, Alzheimer’s disease, and Parkinson’s disease (Block and Hong, 2005; Wang et al., 2007; Stolp and Dziegielewska, 2009).
Compound K [20-O-D-glucopyranosyl-(20S)-protopanaxadiol] is one of the major metabolites of ginseng, which are formed by intestinal bacteria after the oral administration of ginseng extract in humans and rats. The ginseng saponin metabolite, compound K, is absorbed from the gastrointestinal tract to the blood (Akaö et al., 1998). Compound K has a variety of pharmacological activities, including antitumor, antidiabetic, antiinflammatory, and antiplatelet effects (Jia et al., 2010; Radad et al., 2011). Our group has reported that compound K suppresses glioma invasion via the inhibition of MMP-9 expression (Jung et al., 2006). Compound K also inhibited the proliferation of LPS- and/or total saponins exert anti-inflammatory effects in lipopolysaccharide (LPS)- and/or β-amyloid-stimulated microglial cells (Park et al., 2009). Among the individual ginsenosides tested, compound K suppressed LPS-induced NO production. However, the anti-inflammatory effects of compound K in activated microglia and its underlying molecular mechanisms have not been clearly demonstrated. In the present study, we examined the effects of compound K on various inflammatory molecules in LPS-stimulated microglial cells and analyzed the molecular mechanisms. Subsequently, we demonstrated the anti-inflammatory and/or neuroprotective effects of compound K in brain disease models of mice such as sepsis (systemic inflammation) and cerebral ischemia.

Materials and Methods

Reagents. Compound K was prepared as described previously (Joh et al., 2011). In brief, protopanaxadiol-type ginsenosides were incubated with Bacteroides JJY-6, a human intestinal bacterium in a general anaerobic medium for 24 h at 37°C. The incubated medium was extracted with butanol. The supernatant was concentrated in vacuum and processed by using silica gel column chromatography (Joh et al., 2011). We have previously reported that ginseng extracts and total saponins exert anti-inflammatory effects in lipopolysaccharide (LPS)- and/or β-amyloid-stimulated microglial cells (Park et al., 2009). Among the individual ginsenosides tested, compound K suppressed LPS-induced NO production. However, the anti-inflammatory effects of compound K in activated microglia and its underlying molecular mechanisms have not been clearly demonstrated. In the present study, we examined the effects of compound K on various inflammatory molecules in LPS-stimulated microglial cells and analyzed the molecular mechanisms. Subsequently, we demonstrated the anti-inflammatory and/or neuroprotective effects of compound K in brain disease models of mice such as sepsis (systemic inflammation) and cerebral ischemia.

All of the reagents used for cell culture were purchased from Invitrogen (Carlsbad, CA). Antibodies against MAPKs or HO-1 were purchased from Cell Signaling Technology (Danvers, MA). All other chemicals were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise stated.

Microglial Cell Cultures. Immortalized murine BV2 microglial cells (Bocchini et al., 1992) were grown and maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal bovine serum, streptomycin (10 μg/ml), and penicillin (10 U/ml) at 37°C. Primary microglial cells were cultured from the cerebral cortices of 1- to 2-day-old Sprague-Dawley rat pups (Orient Bio Inc., Seongnam, Republic of Korea) as described previously (Park et al., 2009). The purity of microglial cultures was >95%, which was determined by isoelectric B4 staining (data not shown).

Measurement of Cytokine, Nitrite, and Intracellular ROS Levels. Microglial cells (1 × 10⁶ cells per well in a 24-well plate) were pretreated with compound K (25, 50, and 75 μM) for 30 min and stimulated with LPS (0.1 μg/ml). The supernatants of the cultured microglia were collected 24 h after LPS stimulation, and the concentrations of TNF-α and IL-1β were measured by an enzyme-linked immunosorbent assay. Accumulated nitrite was measured in the cell supernatant by using the Griess reagent (Promega, Madison, WI). The intracellular accumulation of ROS was measured with H2-di-chlorofluorescein diacetate (Sigma-Aldrich) by modifying a previously reported method (Qin et al., 2005).

RT-PCR. BV2 cells (7.5 × 10⁵ cells on a 6-cm dish) and rat primary microglia (7 × 10⁶ cells on a 6-cm dish) were treated with LPS in the presence of compound K (25, 50, and 75 μM), and total RNA was extracted with TRI reagent (Sigma-Aldrich). For RT-PCR, total RNA (1 μg) was reverse-transcribed in a reaction mixture containing 1 U RNase inhibitor, 500 ng of random primers, 3 mM MgCl₂, 0.5 mM dNTP, 1× RT buffer, and 1 U reverse transcriptase (Promega). The synthesized cDNA was used as a template for the PCR by using GoTaq polymerase (Promega) and primers (Table 1).

Electrophoretic Mobility-Shift Assay. Nuclear extracts from treated microglia were prepared as described previously (Woo et al., 2003). The double-stranded DNA oligonucleotides containing the NF-xB, AP-1, ARE, or CRE consensus sequences (Promega) were end-labeled by [γ-32P]ATP. EMSA was performed by using 30,000 to 50,000 cpm of labeled probe and nuclear proteins (5 μg) in a final volume of 20 μl of 12.5% glycerol and 12.5 mM HEPES, pH 7.9, 4 mM Tris-HCl, pH 7.9, 60 mM KCl, 1 mM EDTA, and 1 mM dithiothreitol with 1 μg of poly(dI-dC) as nonspecific competitor. The reaction was incubated at room temperature for 20 min. The DNA protein was resolved on high ionic strength, nondenaturing 6% polyacrylamide gel followed by autoradiography with an intensifying screen. For the supershift assay, antibodies to the p65 or p50 subunits of NF-xB (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were coincubated with the nuclear protein in the reaction mixture for 30 min at 4°C before adding the radiolabeled probe.

Transient Transfection and Luciferase Assay. Transfection of the reporter genes into BV2 cells was performed by using Geneporter 2 transfection reagent (Gene Therapy Systems Inc., San Diego, CA). The NF-xB reporter plasmid contains three copies of the xB-binding sequence fused to the firefly luciferase gene (Clontech, Mountain View, CA).

TABLE 1

<table>
<thead>
<tr>
<th>Primers used</th>
<th>Forward Primer, 5’ → 3’</th>
<th>Reverse Primer, 5’ → 3’</th>
<th>Size (base pairs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>iNOS</td>
<td>CTCCTCCGAGAGTTCTGCGAGACGC</td>
<td>GCTTTCAGAGCTCTGCTGCTG</td>
<td>450</td>
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<tr>
<td>TNF-α</td>
<td>CTTGATTCTCAAGCCTCTCTCT</td>
<td>CTCGACATAGGATAAAAT</td>
<td>354</td>
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<tr>
<td>IL-1β</td>
<td>GCAGACAGTTCTCCTCAAGACTAG</td>
<td>CATGAGTTAGGAGAGCTCTGC</td>
<td>447</td>
</tr>
<tr>
<td>IL-6</td>
<td>CACCCTTCTGAGGACGAGCTTT</td>
<td>CAGGCTTCTCTGAGGAGAGA</td>
<td>395</td>
</tr>
<tr>
<td>MCP-1</td>
<td>ACTGAAACAGCAGCTCTTCTCT</td>
<td>CTTCTTGGGAGCTGAGACAG</td>
<td>276</td>
</tr>
<tr>
<td>MMP-3</td>
<td>ATGGAGAGAGGTTCTCTGCTCAG</td>
<td>GGCAGCATCATCATTCTACAG</td>
<td>375</td>
</tr>
<tr>
<td>MMP-9</td>
<td>GTATCCCACCTACCTAAGAAC</td>
<td>AGGAGAAAGGAGCTGAGAGG</td>
<td>420</td>
</tr>
</tbody>
</table>
CA). The ARE-luciferase reporter plasmid contains enhancer 2 (E2) and a minimal promoter sequence of the mouse HO-1 gene fused to the luciferase gene (So et al., 2006). BV2 cells (2 × 10⁵ cells per well in a 12-well plate) were transfected with 1 μg of the reporter construct mixed with Geneporter. After 48 h, cells were harvested and luciferase assay was performed as described previously (Woo et al., 2003). To determine the effect of compound K on reporter gene activity, cells were pretreated with the agent before treatment with LPS (0.1 μg/ml) and incubated for 6 h before harvesting cells.

In Vivo Administration of Compound K. Experiments were performed in male C57BL/6 mice (10–11 weeks old; Orient Bio Inc., Seongnam, Republic of Korea). All experiments were performed in accordance with the National Institutes of Health (Institute of Laboratory Animal Resources, 1996) and Ewha Womans University guidelines for laboratory animals’ care and use, and the study was approved by the Institutional Animal Care and Use Committee at the Medical School of Ewha Womans University. Compound K was dissolved in 3% cremophore (Sigma-Aldrich) in normal saline. Mice were randomly divided into control and treatment groups, and vehicle (3% cremophore in normal saline) or compound K (30 mg/kg i.p.) was administered 4 days before LPS (5 mg/kg i.p.) treatment or transient MCAO. The doses of LPS and compound K were based on a previous study (Park et al., 2009).

Transient Middle Cerebral Artery Occlusion. Procedures for transient MCAO have been established and published previously (Shin et al., 2009). In brief, mice were anesthetized, and a fiber optic probe was attached to the right parietal bone (2 mm posterior and 5 mm lateral to the bregma) and connected to a laser-Doppler flowmeter (Periflux System 5010; Perimed, Järfälla, Sweden). Cerebral blood flow (CBF) was continuously recorded during MCAO and reperfusion periods using a computer-based data acquisition system (Perisoft; Peri Laboratory Animal Resources, 1996) and Ewha Womans University. Only animals that exhibited a reduction in CBF ≤ 85% during 80% after 10 min of reperfusion were used in the study. An animal was calculated.

Infarct Volume Measurement. Infarct volume was measured according to procedures described previously (Lin et al., 1993). Mice were sacrificed 3 days after MCAO, and brains were removed, frozen, and sectioned (30 μm thick) by using a cryostat. Brain sections were collected serially at 600-μm intervals and stained with cresyl violet. Infarct volume was determined by using an image analyzer (Axiovision LE 4.1; Carl Zeiss GmbH, Jena, Germany). Values were reported after correcting for postischemic swelling as described previously (Lin et al., 1993).

Immunohistochemistry. Three hours after LPS treatment or 24 h after MCAO, the animals were anesthetized with sodium pentobarbital (120 mg/kg i.p.) and perfused transcardially with normal saline containing heparin (5 U/ml), followed by 4% formaldehyde in 0.1 M sodium phosphate buffer, pH 7.2. The brains were removed and incubated overnight in fixatives and stored with cresyl violet. Immunohistochemistry. Infarct volume was determined by using an image analyzer (Axiovision LE 4.1; Carl Zeiss GmbH, Jena, Germany). Values were reported after correcting for postischemic swelling as described previously (Lin et al., 1993).

Results

Compound K Inhibited iNOS, Cytokine, MCP-1, and MMP-3/9 Expression in LPS-Stimulated Microglial Cells. To investigate the anti-inflammatory effects of compound K, BV2 cells and primary microglia were treated with compound K for 30 min before stimulation with LPS. As shown in Fig. 2A, compound K inhibited LPS-induced production of NO, TNF-α, and IL-1β in a dose-dependent manner. Subsequent RT-PCR analysis revealed that compound K suppressed iNOS, TNF-α, and IL-1β expression at the mRNA level (Fig. 2B). Compound K also inhibited the expression of IL-6, MCP-1, MMP-3, and MMP-9, which also play an important role in LPS-induced inflammatory reactions.

Compound K Inhibited NF-κB and AP-1 Activities, while It Increased Nuclear Protein Binding to CRE. To study the anti-inflammatory mechanism of compound K, we examined the effect of compound K on NF-κB and AP-1, which are important transcription factors modulating cytokine, MMP/MCP-1, and iNOS gene expression in microglia (Lee and Kim, 2009; Smale, 2010). As shown in Fig. 3, stimulation of BV2 cells with LPS resulted in strong NF-κB and AP-1 binding, which was significantly inhibited by compound K. In addition, compound K inhibited NF-κB-mediated transcriptional activity, as shown by the k-luc reporter gene assay (Fig. 3B). Next, we examined the effect of compound K on CREB, which is known to be involved in anti-inflammatory mechanisms of activated microglia (Woo et al., 2003; Jung et al., 2010). As shown in Fig. 3D, compound K significantly enhanced nuclear protein binding to CRE. The data
collectively suggest that compound K may inhibit the expression of various inflammatory molecules by modulating NF-κB, AP-1, and CREB.

**Compound K Inhibits LPS-Induced ROS Production, Phosphorylation of p47phox of NADPH Oxidase, and Three Types of MAPKs.** Excessive ROS generation by microglia contributes to the aggravation of neuronal damage after a stroke (Sorce and Krause, 2009). In addition, ROSs are known as second messengers in inflammatory reactions (Sayre et al., 2008). In the present study, compound K significantly attenuated LPS-induced ROS production in BV2 cells and primary microglia (Fig. 4, A and B). Moreover,
Compound K suppressed the phosphorylation of p47phox, a major component of NADPH oxidase (Nox2) responsible for microglial ROS release (Fig. 4C) (Sorce and Krause, 2009). Thus, the antioxidant effect of compound K may partly attribute to reduced NADPH oxidase activity. Furthermore, compound K inhibited the LPS-induced phosphorylation of three types of MAPKs, which are also important upstream signaling molecules in inflammatory reactions (Fig. 4D).

**Compound K Up-Regulated HO-1 Expression via ARE.** Next, we examined the effect of compound K on the expression of HO-1, which is known as a key molecule in the resolution of oxidative stress and inflammation (Min et al., 2006). RT-PCR and Western blot analyses showed that compound K increased HO-1 expression at the mRNA and protein levels (Fig. 5, A and B). Because the AREs on the HO-1 promoter are critical for HO-1 transcription, we examined the effect of compound K on nuclear protein binding to ARE. As shown in Fig. 5C, compound K significantly increased the ARE-nuclear protein complex. Moreover, compound K increased ARE-driven luciferase activity (Fig. 5D).

**Compound K Repressed Inflammatory Responses in the Septic Brain of Mice.** To determine whether compound K also reduced microglial activation in vivo, we measured the immunoreactivity of Iba1, which is a marker for activation of microglia (Ito et al., 2001), in the mouse cortex and striatum 3 h after systemic administration of LPS. In the brain of LPS-injected mice, the number of Iba1-positive cells with a densely stained round shape, indicative of activated cells, was increased compared with controls. However, pretreatment with compound K (30 mg/kg) reduced the number of activated microglia, as seen after the quantification of activated microglia in both the cortex and the striatum (p < 0.01 compared with vehicle; Fig. 6A). In addition, compound K significantly reduced the expression of LPS-induced IL-1β and TNF-α protein in the cortex 6 h after LPS injection (Fig. 6B).

**Neuroprotective and Anti-Inflammatory Effects of Compound K on Ischemic Brain Injury in Mice.** Inflammation plays important roles in ischemic brain damage (Wang et al., 2007). Based on the above results, we further examined the therapeutic potential of compound K on ischemic stroke induced by transient MCAO in mice. As shown in Fig. 7A, pretreatment of compound K (30 mg/kg) significantly decreased the total infarct volume compared with vehicle treatment (46% reduction; p = 0.012). The reduction in infarct volume was prominent in the cortex (~56% compared with vehicle; p = 0.049), the area of ischemic penumbra, but not in the striatum, which is the ischemic core. These findings are consistent with previous reports showing that the penumbra is a salvageable area by neuroprotective therapies (Ginsberg, 2003; Shin et al., 2009, 2010b). Next, we examined the effects of compound K on microglial activation in the ischemic brain by using Iba1 antibody. Iba1 expression was assessed by immunofluorescence labeling in the ipsilateral brain 24 h after MCAO. As shown in Fig. 7B, MCAO led to the appearance of numerous densely stained, activated microglial cells in the cortex and striatum in vehicle-treated mice. Pretreatment of compound K significantly reduced the number of activated microglia in the cortex, but not in the striatum (Fig. 7B). The results are correlated with the data in Fig. 7A, which show a reduction in infarct volume in the cortex, but not in the striatum. The data suggest that micro-
glial inactivation is at least partly responsible for the neuroprotective effects of compound K against an ischemic insult.

Discussion

In the present study, we demonstrate the anti-inflammatory effects of compound K in activated microglia in vitro and in vivo. Compound K suppressed the expression of various inflammatory molecules in LPS-stimulated BV2 cells and primary microglia. The anti-inflammatory effects of compound K were supported by two animal models: systemic inflammation and cerebral ischemia. Compound K suppressed microglial activation induced by systemic LPS administration in vivo. In addition, compound K showed neuroprotective effects with reduction of microglial activation in the ischemic brains of mice. Detailed mechanistic studies using in vitro cell culture systems revealed that compound K suppresses inflammatory molecules via modulating ROS, MAPKs, NF-κB/AP-1, and HO-1/ARE signaling pathways.

A number of studies have reported on the beneficial effects of ginsenosides (ginseng saponins) in the central nervous system. Ginsenosides potentiate brain functions by promoting neurogenesis and affecting neurotransmission (Liu et al., 2007; Shen and Zhang, 2007). Ginsenosides, such as Rh1, Rh2, Rb1, and Rg1, support memory and learning (Wang et al., 2007; Shen and Zhang, 2007). Ginsenosides, such as Rh1, Rb1, and Rg1, support memory and learning (Wang et al., 2007; Shen and Zhang, 2007). Because Rb1 is metabolized into compound K by intestinal microflora before absorption in the body (Akao et al., 1998; Bae et al., 2004), the in vivo anti-ischemic effect of ginsenoside Rb1 might be caused by the metabolites. In support of this notion, the present study demonstrates that compound K exerts protective effects against ischemic stroke. We found that compound K reduced ischemic brain injury in the cortex, but not in the striatum, which is destined to be the necrotic core (Fig. 7A), indicating that compound K can be a effective therapeutic to rescue the salvageable penumbra from ischemic insults. In addition, we found that compound K repressed microglial activation, which contributes to ischemic brain damage by releasing proinflammatory cytokines and free radicals (Wang et al., 2007). The number of morphologically activated microglia that were detected prominently at 24 h after MCAO (Shin et al., 2010b) was decreased in the ischemic cortex of mice pretreated with compound K (Fig. 7B). The repression of microglia is not likely a result of reduced infarct volume because cortical infarction was not definitely visible at 24 h after MCAO, the time point at which Iba-1 expression was examined. The reason that compound K repressed microglial activation only in the cortex might be explained by the different pathophysiology between the core and the penumbra. It has been shown that molecules released from damaged neurons, such as ATP and glutamate, activate microglia (Volonté et al., 2003), and the activated microglia can again increase neuronal death by releasing cytotoxic agents such as cytokines, ROS, proteases, and glutamate (Kato and Kogure, 1999). Therefore, more prominent activation of microglia in the striatum may indicate more severe cytotoxic responses in the core of infarction, and the dose or treatment timing of compound K may not be enough to repress microglial activation in the core area. Although a direct link between microglial activation and neuroprotection by compound K remains to be further investigated in ischemic stroke models, we suggest that the reduction of infarct volume by compound K is associated with repression of microglial activation, and the data support the concept that the penumbra is a salvageable area by suitable protective drugs.
The effects of compound K on microglial activation were further supported by studies using mice exposed to systemic administration of LPS, an acute model of infection causing inflammation in global area of the brain (Qin et al., 2007; Shin et al., 2010a). Compound K reduced microglial activation not only in the cortex but also in the striatum because there was no regional difference of injury severity between the cortex and the striatum in this model. Furthermore, compound K also reduced the expression of proinflammatory cytokines IL-1β and TNF-α (Fig. 6). Collectively, the data support the view that anti-inflammatory action may be one of the factors contributing to the neuroprotective effects afforded by compound K.

In BV2 microglial cells and primary cultured microglia, compound K inhibited not only the expression of proinflammatory molecules (iNOS, TNF-α, IL-1β, IL-6, MCP-1, and MMP-3/9) but also ROS production. A recent article reported on the in vitro antioxidant properties of compound K (Lee et al., 2011). Compound K exhibits strong radical scavenging activities against 1,1-diphenyl-2-picrylhydrazyl, hydroxyl, superoxide, and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid). Compound K also inhibited lipid peroxidation. Another article reported that compound K regulates zymosan-induced inflammatory signaling through the inhibition of ROS (Cuong et al., 2009). Therefore, the results suggest that compound K may be a useful antioxidant agent against reactive oxygen species.

In the present study, we found that compound K increased HO-1, which is known to have antioxidant, antiapoptotic, and anti-inflammatory functions (Naidu et al., 2009). Previous studies have shown a link between HO-1 activity and reduction in iNOS expression (Min et al., 2006). In addition, HO-1 inhibits LPS-induced TNF-α and IL-1β expression through the suppression of NF-κB (Rushworth et al., 2008). Furthermore, carbon monoxide, one of the products of HO-1, inhibits NADPH oxidase and Toll-like receptor 4, which are involved in LPS signaling (Nakahira et al., 2006). Thus, the induction of HO-1 may provide the basis not only for the

**Fig. 6.** Compound K reduced inflammatory responses in the septic brain of mice. A, immunofluorescence labeling of Iba1 and quantification of the number of activated Iba1-positive cells in the cortex and striatum 3 h after systemic LPS treatment (5 mg/kg). Representative images were obtained from one set of experiments, and three experiments were done independently. The data are the mean ± S.E.M. B, Western blots for IL-1β, TNF-α, and actin in the cortex 6 h after LPS treatment and quantification of IL-1β and TNF-α protein levels with normalization by actin (n = 3 in each group). Values (mean ± S.E.M.) are the ratio versus controls (assigned a nominal value of 1). *, P < 0.05, significantly different from the value in control mice. #, P < 0.05, significantly different from the value in vehicle-treated mice.
anti-inflammatory activity of compound K, but also the antioxidant activity.

When we examined the effects of compound K on various transcription factors involved in the regulation of inflammatory molecules, compound K was shown to inhibit NF-κB and AP-1 activities, while increasing nuclear protein binding to CRE and ARE in LPS-stimulated microglia. Studies indicate that CREB, as well as nuclear factor E2-related factor-2 (ARE binding protein), play a role in the regulation of ROS detoxification (Lee et al., 2009). In addition, the antioxidant enzyme HO-1 has been shown to be under the influence of the CREB/CRE transcriptional pathway (Min et al., 2006; Lee et al., 2009). Therefore, CREB, in concert with HO-1, seems to play an important role in compound K-mediated anti-inflammation/antioxidant effects in microglia.

Another study reported that compound K acts as an agonist of glucocorticoid receptor (GR) and induces tolerance to endotoxin-induced lethal shock (Yang et al., 2008). It was suggested that the therapeutic effect of compound K on lethal sepsis is mediated through the modulation of Toll-like receptor 4-associated signaling via the GR. Thus, GR agonist function might also be related with the anti-inflammatory effects of compound K in our neuroinflammatory model systems. Further studies will be necessary to clarify this issue.

In conclusion, we report for the first time the anti-inflammatory effects of compound K in microglial cell culture systems and two in vivo models of brain disease in which inflammation plays important roles. Furthermore, the neuroprotective effect of compound K was demonstrated in a mouse model of ischemic brain injury. Detailed mechanistic studies indicate that the inhibition of ROS, MAPKs, and NF-κB/AP-1, and the enhancement of the CREB and nuclear factor E2-related factor-2/HO-1 signaling axis, are responsible for the strong anti-inflammatory/antioxidant effects of compound K in activated microglia.

Therefore, compound K is a promising therapeutic agent for the prevention and/or treatment of ischemic brain injuries and other neurodegenerative diseases that are accompanied by microglial activation.

**Authorship Contributions**

*Participated in research design:* E.-M. Park and H.-S. Kim.

*Conducted experiments:* J.-S. Park, Shin, Jung, and E.-M. Park.
Contributed reagents or analytic tools: Hyun, Le, and D.-H. Kim.

Performed data analysis: E.-M. Park and H.-S. Kim.

Wrote or contributed to the writing of the manuscript: J.-S. Park, E.-M. Park, and H.-S. Kim.

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