INTRODUCTION

Astrocytes are the major glial cells in the brain and are implicated in the segregation, maintenance, and support of neurons. Astrocytes also perform a wide range of functions, including guidance of the maturation and migration of neurons during brain development, production of growth factors, maintenance of the integrity of the blood-brain barrier, and participation in the immune and repair responses to disease and brain injury (Sofroniew and Vinters, 2010; Dallerac et al., 2013). In particular, astrocytes are enriched with antioxidant enzymes that enable detoxification and protection of the brain against oxidative stress (Sypin, 2008; Vargas and Johnson, 2009).

The antioxidant responsive element (ARE) is a cis-acting regulatory element on the promoter regions of genes encoding phase II detoxification enzymes and antioxidant proteins (Jaiswal, 2004; Lee and Johnson, 2004). In general, Nrf1 and/or Nrf2 are known to bind to ARE and induce the gene expression of phase II antioxidant enzymes, such as heme oxygenase-1 (HO-1), NAD(P)H:quinone oxidoreductase 1 (NQO1), manganese superoxide dismutase (MnSOD), and catalase (Venugopal and Jaiswal, 1998; Jaiswal, 2004). Thus, many research groups have been exploring natural or synthetic compounds that can enhance antioxidant enzyme expression in normal and/or diseased conditions.

Ginsenoside Rh1, a bacterial metabolite of ginsenoside Rg1, is one of the major saponin components of red ginseng and has a protopanaxatriol structure (Shin et al., 2006; Jung et al., 2010b). Previous studies have reported that Rh1 has anti-inflammatory, antioxidant, anti-allergic, anti-ammestic, and anti-aging effects (Park et al., 2004; Cheng et al., 2005; Zhu et al., 2009). Rh1 inhibits the IgE-induced cutaneous anaphylaxis reaction via inhibition of NF-κB (Park et al., 2004). In addition, Rh1 ameliorates oxazolone-induced skin dermatitis by increasing Foxp3 expression and Treg cell differentiation (Zheng et al., 2011). A recent study reported that Rh1 ameliorates TNBS-induced colitis by inhibiting LPS-TLR4 binding on...
macrophages and modulating Th17/Treg balance (Lee et al., 2015b). Rh1 potentiates the anti-inflammatory effects of dexamethasone in chronic inflammatory disease by reversing dexamethasone-induced resistance (Li et al., 2014). Our group recently reported that Rh1 suppresses neuroinflammation by modulating protein kinase A and HO-1 expression in activated microglia (Jung et al., 2010b). Moreover, we found that Rh1 inhibits the expression of matrix metalloproteinases and the in vitro invasion/migration of human astroglioma cells (Jung et al., 2013).

Despite a variety of therapeutic effects of Rh1 in the brain and peripheral systems, the antioxidant effect of Rh1 in astrocytes has not been reported. In this study, we found that Rh1 exerted antioxidant and cytoprotective effects in hydrogen peroxide-treated rat primary astrocytes, and increased phase II antioxidant enzyme gene expression by upregulation of the Nrf2/ARE axis. Furthermore, we demonstrated that MAP kinases are important in HO-1 expression, and act by modulating ARE-mediated transcriptional activity.

**MATERIALS AND METHODS**

**Reagents**

Ginsenoside Rh1[6-O-β-D-glucopyranosyl-20(S)-protopanaxatriol], a bacterial metabolite of Rg1, was isolated according to previous methods (Shin et al., 2006). The structure of Rh1 is shown in Fig. 1A. All reagents used for cell culture containing penicillin/streptomycin, trypsin, and minimal essential medium were purchased from Invitrogen (Carlsbad, CA, USA). TRI reagent was purchased from Sigma Chemical Co. (St Louis, MO, USA). Antibodies against the phospho-/total form of p38 MAPK, ERK1/2, and SAPK/JNK were purchased from Cell Signaling Technology (Beverley, CA, USA). All other chemicals were obtained from Sigma-Aldrich, unless stated otherwise.

**Rat primary astrocyte cell culture**

Rat primary astrocyte cultures were prepared from mixed glial cultures using a previous method with modifications (Park et al., 2011). In brief, after cortices were dissected from 2-day-old rats, cells were dissociated by pipetting and resuspended in minimal essential medium containing 10% fetal bovine serum, streptomycin (10 μg/mL), penicillin (10 U/mL), 2 mM glutamine, and 10 mM HEPES. Cell suspensions were plated on poly-D-lysine (1 μg/mL)-coated T75 flasks and incubated for 7–10 days. After the primary cultures reached confluence, the culture flasks were shaken at 280 rev/min for 16 h to remove microglia and oligodendrocytes. The purity of the astrocyte-enriched cultures (>95%) were confirmed by staining with antibodies against the astrocyte-specific marker glial fibrillary acidic protein.

**Intracellular ROS measurement and cell viability test**

Intracellular accumulation of ROS was measured using a modification of previously described methods (Qin et al., 2005). In brief, astrocytes were stimulated with H2O2 for 1 h, then stained with 50 mM H2DCF-DA in HBSS buffer for 30 min at 37°C. DCF fluorescence intensities were measured at excitation and emission wavelengths of 485 and 535 nm, respectively, using a fluorescence plate reader (Molecular Devices, CA, USA). Cell viability was determined using the MTT reduction assay as previously described (Park et al., 2009).

**Western blot analysis**

Cells were appropriately treated and total cell lysates were
prepared as described in a previous study (Park et al., 2011). The proteins (20-100 μg) were heated with 4×SDS sample buffer and separated by SDS-PAGE gel electrophoresis and transferred to nitrocellulose membranes (GE Healthcare, Chalfont, Buckinghamshire, UK). The membranes were blocked with 5% bovine serum albumin in 10 mM Tris-HCl containing 150 mM NaCl and 0.5% Tween-20 (TBST) and then incubated with primary antibodies (1:1000) that recognize the phospho-or the total forms of MAP kinases. After thoroughly washing with TBST, horseradish peroxidase-conjugated secondary antibodies (1:2000 dilution in TBST; New England Biolabs, Ipswich, MA, USA) were applied and the blots were developed using an enhanced chemiluminescence detection kit (Thermo Fisher Scientific, Waltham, MA, USA).

RT-PCR
Total cellular RNA was extracted from appropriately treated primary astrocytes with TRI reagent according to the manufacturer's protocol. For RT-PCR, total RNA (1 μg) was reverse-transcribed in a reaction mixture that contains 1 U RNase inhibitor, 500 ng random primers, 3 mM MgCl2, 0.5 mM dNTP, and 10 U reverse transcriptase (Promega, Madison, WI, USA). The synthesized cDNA was used as a template for PCR reaction using GoTaq polymerase (Promega) and primers, as shown in Table 1.

Electrophoretic mobility shift assay (EMSA)
Nuclear extracts were prepared from astrocytes as previously described (Lee et al., 2015a). The double-stranded DNA oligonucleotides containing the ARE consensus sequences (Promega) were end-labeled by [γ-32P] ATP. Five micrograms of the nuclear proteins were incubated with 32P-labeled ARE probes on ice for 30 min and resolved on a 5% acrylamide gel as previously described (Lee et al., 2015a).

Transient transfection and luciferase assays
Rat primary astrocytes were plated in 12 well plate at the density of 2.5×105 cells, and transfected with 0.5 μg of plasmid DNA using Convoy™ Platinum transfection reagent (CellTAsGen, Seoul, Korea). To determine the effect of Rh1 on ARE promoter activity, cell were treated with Rh1 and incubated for 16 h prior to harvesting cells and luciferase assay was performed as previously described (Park et al., 2011).

Statistical analysis
Unless otherwise stated, all experiments were performed with triplicate samples and repeated at least three times. The data are presented as mean ± S.E.M. and statistical comparisons between groups were performed by using one-way analysis of variance, followed by Newman-Keuls test. A p-value < 0.05 was considered significant.

RESULTS
Rh1 inhibited reactive oxygen species (ROS) production and cell death in H2O2-treated astrocytes
To determine whether Rh1 exerts antioxidant effects in astrocytes, intracellular ROS-scavenging activities of Rh1 were measured in H2O2-treated rat primary astrocytes. As shown in Fig. 1B, Rh1 significantly inhibited intracellular ROS production. In addition, Rh1 attenuated H2O2-induced cell death, as shown by MTT assay data (Fig. 1C). The results suggest that Rh1 may produce cytoprotective effects via the inhibition of ROS production.

Rh1 increased the expression of phase II antioxidant enzymes in astrocytes
Phase II antioxidant enzymes, such as HO-1, NQO-1, superoxide dismutase-2 (SOD-2), and catalase, are important components of the cellular defense mechanism against oxidative stress (Zhang et al., 2013). Thus, we investigated whether or not Rh1 induced antioxidant enzyme expression in rat primary astrocytes. Western blot analysis revealed that Rh1 induced the protein expression of HO-1, NQO-1, SOD-2, and catalase (Fig. 2A, B). Furthermore, Rh1 increased the expression of those enzymes at the mRNA level as shown by the RT-PCR analysis (Fig. 2C, D). Interestingly, Rh1 (300 μM) induced the mRNA and protein expression of antioxidant enzymes at 1 h, the level of which was increased up to 6-12 h. In case of catalase, however, the protein expression pattern after 9 h was not coincident with mRNA expression, which may be due to posttranscriptional regulation.

Rh1 increased the nuclear translocation and DNA binding of Nrf2/c-Jun to ARE, and increased ARE-mediated transcriptional activities in astrocytes
We have recently demonstrated that Nrf2 and c-Jun bind to ARE and coordinately regulate HO-1 expression in astrocytes (Park et al., 2011; Park and Kim, 2014). Thus, we examined the effects of Rh1 on Nrf2 and c-Jun translocation to the nucleus. Western blot analysis showed that Rh1 increased the protein levels of Nrf2 and c-Jun in nuclear extracts of astrocyte cells (Fig. 3A). Next, we performed EMSA to determine whether Rh1 increases nuclear factor binding to ARE. As shown in Fig. 3B, Rh1 increased the levels of the ARE-nuclear protein binding complex. In addition, Rh1 increased ARE-mediated transcriptional activities, as shown by ARE-luc reporter gene assay (Fig. 3C). In addition, Rh1 increased the activities of HO-E1-luc, which contains three ARE sites within HO-1 enhancer 1 (Fig. 3D). The data suggest that Rh1 increases the expression of antioxidant enzyme genes, such as HO-1, by enhancing the binding of the transcription factor Nrf2/c-Jun to ARE.

Table 1. Primer sequences for RT-PCR
<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer (5’→3’)</th>
<th>Reverse primer (5’→3’)</th>
<th>Size (bp)</th>
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<tbody>
<tr>
<td>Catalase</td>
<td>CCTGACATGGTCTGGGACTT</td>
<td>CAAGTTTGTAGGCCCTGGT</td>
<td>201</td>
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<tr>
<td>HO-1</td>
<td>TGTCACCCTGCTGGACCTC</td>
<td>ATACCCCCTACCTGGGTGAC</td>
<td>209</td>
</tr>
<tr>
<td>NQO1</td>
<td>ATCACCAGGTCGGAGCCTTC</td>
<td>GCCATGAAAGGGTCTGGT</td>
<td>210</td>
</tr>
<tr>
<td>SOD-2</td>
<td>GGGCAAGGAGGGATTTACAA</td>
<td>GAACCTGGACTCCCACAGA</td>
<td>216</td>
</tr>
<tr>
<td>GAPDH</td>
<td>GTGCTGAGTATGTCCTGGAGTC</td>
<td>ACAGTCTTCGAGSTGGAGTC</td>
<td>395</td>
</tr>
</tbody>
</table>
Fig. 2. Effect of Rh1 on phase II antioxidant enzyme expression in rat primary astrocytes. (A) Cells were incubated with 300 μM Rh1 for the indicated time points and western blot analysis was performed using antibodies against HO-1, NQO-1, SOD-2, and catalase. The data are representative of three independent experiments. (B) Quantification of western blot data. The protein expression was normalized by β-actin and the fold induction of Rh1-treated samples versus control cells was indicated. Values are the mean ± S.E.M. of three independent experiments. *p<0.05; compared with the control sample. (C) The mRNA levels of HO-1, NQO-1, SOD-2, and catalase were determined by RT-PCR analysis. (D) Quantification data. Values are the mean ± S.E.M. of three independent experiments. *p<0.05; compared with the control sample.
MAPK signaling pathways are involved in HO-1 expression by modulating ARE-mediated transcriptional activities. Previous studies have reported that antioxidant enzyme gene expression is under the control of MAPK signaling pathways in many cell types (Niture et al., 2010; Park et al., 2011). To determine the signaling pathways involved in the expression of antioxidant enzyme genes such as HO-1 in Rh1-treat-
rh1 increased the phosphorylation of three types of MAPKs. Treatment of the cells with each signaling pathway-specific inhibitor showed that HO-1 expression was inhibited by MAPKs inhibitors (Fig. SA, B). In addition, MAPK inhibitors significantly inhibited the reporter gene activity of ARE-luc and HO-E1-luc. The results collectively indicate that three types of MAPKs are involved in HO-1 upregulation by modulating ARE in Rh1-treated astrocytes.

**DISCUSSION**

In the present study, we demonstrated that ginsenoside Rh1 inhibited astroglial cell death induced by H2O2, with a reduction of intracellular ROS levels (Fig. 1). The results suggest that the antioxidant effect of Rh1 may contribute to protection of the astrocytes against oxidative stress. In accordance with this, Rh1 increased the expression of antioxidant enzyme genes, such as HO-1, NQO-1, SOD-2, and catalase in rat primary astrocytes (Fig. 2). In addition, Rh1 increased Nrf2/c-Jun binding to ARE and subsequent transcriptional activities (Fig. 3). Finally, MAPK signaling pathways were determined to be involved in HO-1 expression in Rh1-treated astrocyte cells (Fig. 4, 5).

A number of studies have reported that the activation of MAPK signaling pathways are linked to the upregulation of phase II antioxidant enzymes (Jaiswal, 2004; Alam and Cook, 2007; Niture et al., 2010). Oxidative stress activates MAPKs, which subsequently phosphorylate Nrf2 and facilitate Nrf2 release from its cytosolic inhibitor Keap1. Then, Nrf2 is translocated into the nucleus and binds to ARE, inducing the expression of downstream antioxidant genes. Alternatively, a recent study suggested that MAPK increases Nrf2 protein synthesis rather than directly modulating Nrf2 activity (Sun et al., 2009).

In the present study, we demonstrated that Rh1 increased the phosphorylation of three types of MAPKs, and that their specific inhibitors blocked Nrf2/ARE activation and subsequent
HO-1 expression in Rh1-treated astrocytes, suggesting that MAPK phosphorylation by Rh1 plays an important role in Nrf2/ARE activation. Further studies are necessary to determine whether MAPKs also governs the expression of other phase II antioxidant enzymes in addition to that of HO-1.

We previously demonstrated that Rh1 inhibits microglial activation in the brains of mice with LPS-induced systemic inflammation by directly penetrating the blood-brain barrier (Jung et al., 2010b). We also showed that Rh1 increases the viability of neighboring neuronal cells by inhibiting microglial activation. Moreover, Rh1 suppresses iNOS gene expression in IFNγ-stimulated microglial cells via inhibition of the JAK/STAT and ERK signaling pathways (Jung et al., 2010a). Several studies by other groups have also reported on the neuroprotective effects of Rh1. Rh1 increases hippocampal excitability and improved memory in rat and mouse brains (Wang et al., 2009). In addition, long-term administration of Rh1 enhances learning and memory by promoting cell survival and brain-derived neurotrophic factor expression in the mouse hippocampus (Hou et al., 2014). Therefore, these findings collectively suggest that Rh1 may have therapeutic potential for various neurodegenerative disorders that are accompanied by oxidative stress, as well as neuroinflammation.

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REFERENCES


