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Protopanaxatriol Ginsenoside Rh1 Upregulates Phase II Antioxidant Enzyme Gene Expression in Rat Primary Astrocytes: Involvement of MAP Kinases and Nrf2/ARE Signaling

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Abstract

Oxidative stress activates several intracellular signaling cascades that may have deleterious effects on neuronal cell survival. Thus, controlling oxidative stress has been suggested as an important strategy for prevention and/or treatment of neurodegenerative diseases. In this study, we found that ginsenoside Rh1 inhibited hydrogen peroxide-induced reactive oxygen species generation and subsequent cell death in rat primary astrocytes. Rh1 increased the expression of phase II antioxidant enzymes, such as heme oxygenase-1 (HO-1), NAD(P)H:quinone oxidoreductase 1, superoxide dismutase-2, and catalase, that are under the control of Nrf2/ARE signaling pathways. Further mechanistic studies showed that Rh1 increased the nuclear translocation and DNA binding of Nrf2 and c-Jun to the antioxidant response element (ARE), and increased the ARE-mediated transcription activities in rat primary astrocytes. Analysis of signaling pathways revealed that MAP kinases are important in HO-1 expression, and act by modulating ARE-mediated transcriptional activity. Therefore, the upregulation of antioxidant enzymes by Rh1 may provide preventive therapeutic potential for various neurodegenerative diseases that are associated with oxidative stress.

Key Words: Astrocytes, Ginsenoside Rh1, Antioxidant enzyme, MAPK-Nrf2 signaling

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-amnestic, 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

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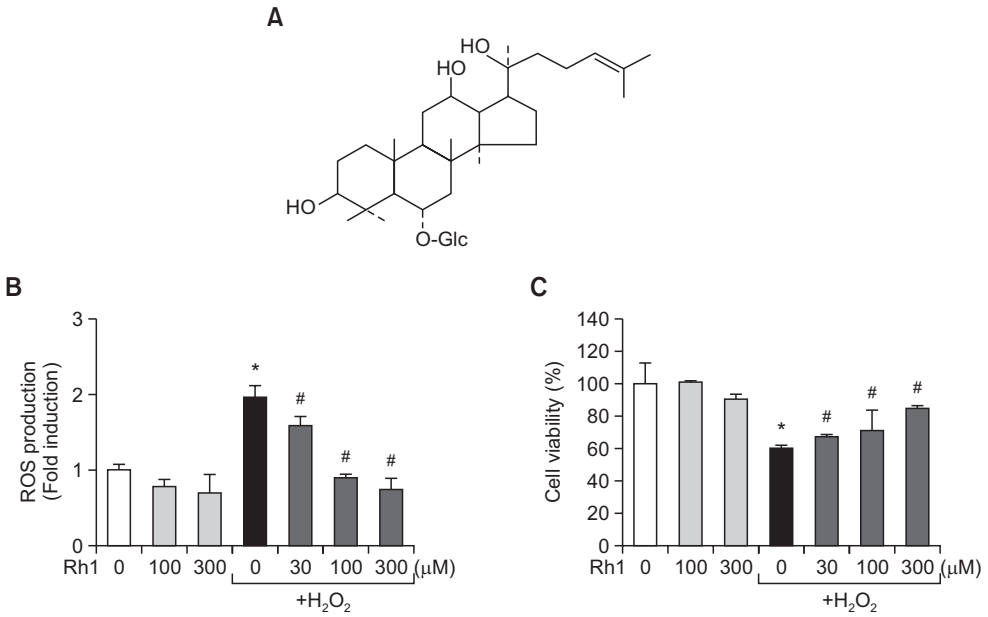

Fig. 1. Effects of Rh1 on reactive oxygen species (ROS) production and cell viability in astrocytes. (A) Chemical structure of protopanaxatriol ginsenoside Rh1. (B) Rat primary astrocytes were pre-treated with Rh1 for 1 h, followed by treatment with H_2O_2 (500 μM) for 30 min. Then, intracellular ROS levels were measured by the DCF-DA method. (C) The viability of rat primary astrocytes was determined by the MTT assay. Values correspond to the mean ± S.E.M. of three independent experiments. *p<0.05; compared with the control cells. #p<0.05; compared with the cells treated with H_2O_2.

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

GinsenosideRh1[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 form Cell Signaling Technology (Beverley, CA, USA). All other chemicals were obtained from Sigma-Aldrich, unless stated otherwise.

Rat primary astrocyte cell culture

Rat primary astrocytes 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 H_2O_2 for 1 h, then stained with 50 mM H_2DCF-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

Table 1. Primer sequences for RT-PCR

Gene	Forward primer (5'→3')	Reverse primer (5'→3')	Size (bp)
Catalase	CCTGACATGGTCTGGGACTT	CAAGTTTTTGATGCCCTGGT	201
HO-1	TGTCACCCTGTGCTTGACCT	ATACCCGCTACCTGGGTGAC	209
NQO1	ATCACCAGGTCTGCAGCTTC	GCCATGAAGGAGGCTGCTGT	210
SOD-2	GGCCAAGGGAGATGTTACAA	GAACCTTGGACTCCCACAGA	216
GAPDH	GTGCTGAGTATGTCGTGGAGTC	ACAGTCTTCTGAGTGGCAGTCA	395

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 $MgCl_2$, 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-^{32}P] ATP. Five micrograms of the nuclear proteins were incubated with ^{32}P-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 2.5×10^5 cells, and transfected with 0.5 μg of plasmid DNA using Convoy^{Tm} Platinum transfection reagent (CellTA-Gen, 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 H_2O_2-treated astrocytes

To determine whether Rh1 exerts antioxidant effects in astrocytes, intracellular ROS-scavenging activities of Rh1 were measured in H_2O_2-treated rat primary astrocytes. As shown in Fig. 1B, Rh1 significantly inhibited intracellular ROS production. In addition, Rh1 attenuated H_2O_2-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.

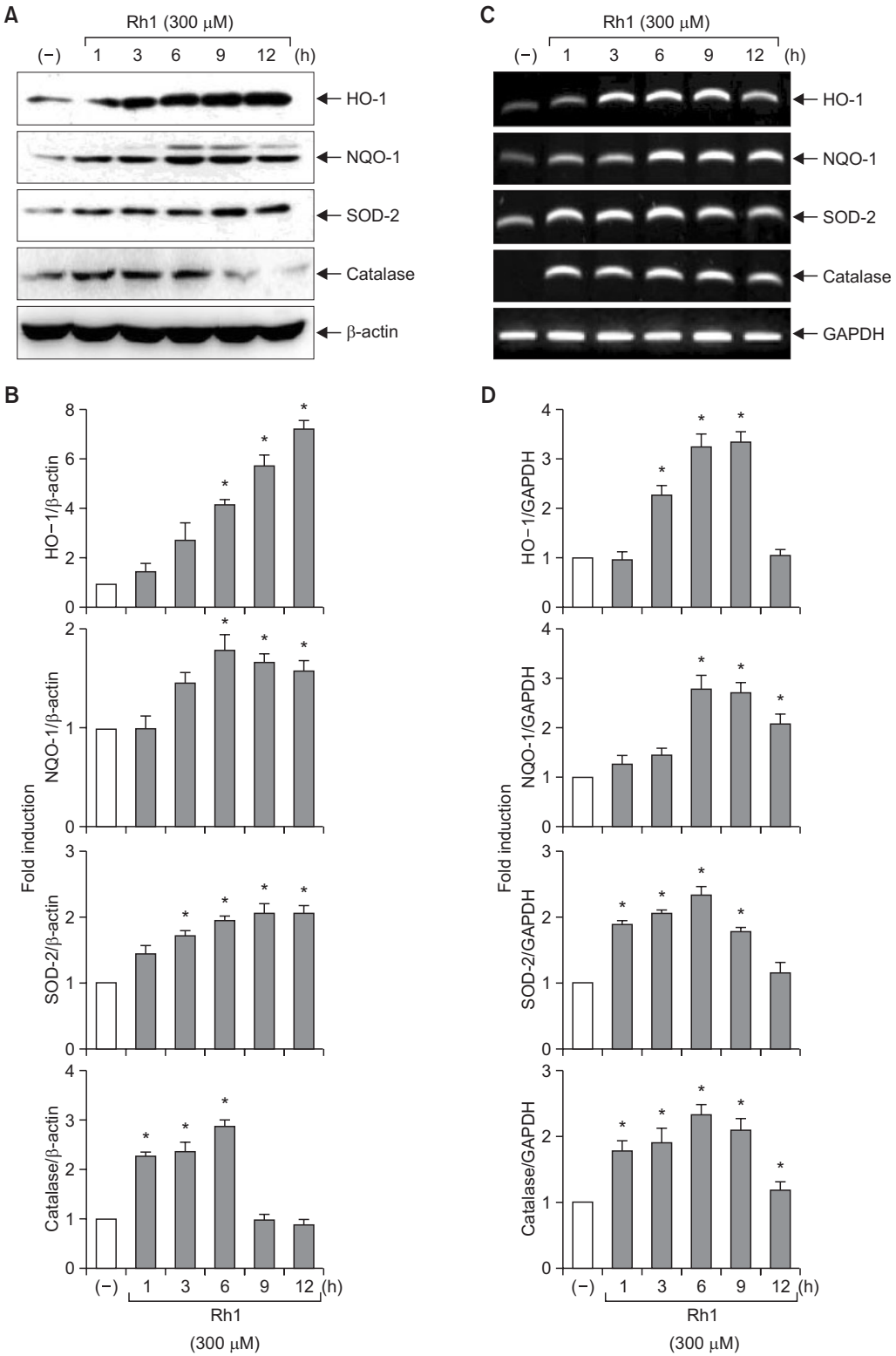

Fig. 2. Effect of Rh1 on phase II antioxidant enzyme expression in rat primary astrocytes. (A) Cells were incubated with 300 μM Rh1 for the indicated time points and western blot analysis was performed using antibodies against HO-1, NQO-1, SOD-2, and catalase. The data are representative of three independent 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.

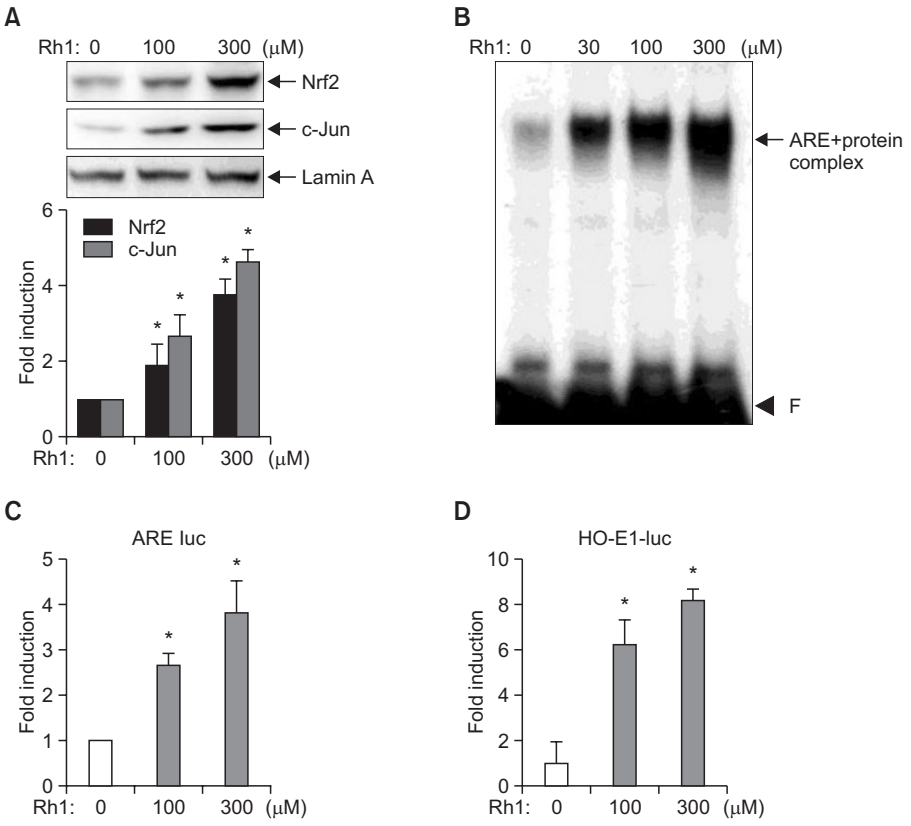

Fig. 3. Effect of Rh1 on nuclear translocation and DNA binding of Nrf2/c-Jun to ARE, and on ARE-mediated transcriptional activities. Nuclear extracts were prepared from primary astrocytes after treatment with Rh1 for 1 h, and western blot and EMSA were then performed. (A) Western blot analysis showed that Rh1 significantly increased the nuclear translocation of Nrf2 and c-Jun. (B) EMSA showed that Rh1 enhanced nuclear protein binding to ARE. The arrow indicates the ARE-nuclear protein complex. 'F' indicates free probe. (C,D) Primary astrocytes were transfected with ARE-luc (C) or HO-E1-luc (D) and treated with Rh1. After 24 h, cells were harvested and the luciferase assay was performed. The values correspond to the mean ± S.E.M. of three independent experiments. **p*<0.05; compared with the control sample.

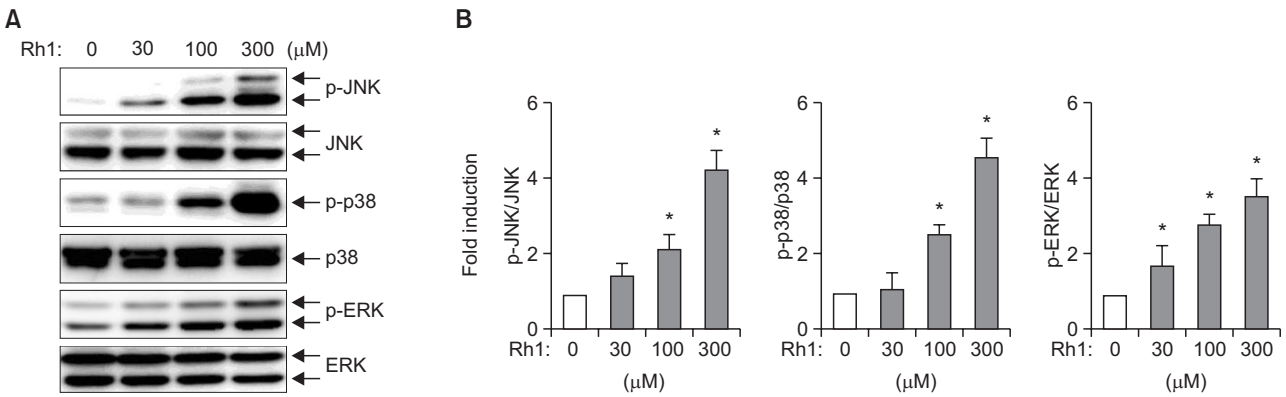

Fig. 4. Effect of Rh1 on the phosphorylation of MAP kinases in rat primary astrocytes. (A) Cell extracts were prepared from astrocytes treated with the indicated doses of Rh1 for 30 min and subjected to immunoblot analysis using antibodies against phospho- or total-forms of three types of MAP kinase (p38 MAPK, ERK1/2, and JNK). The blots are representative of three independent experiments. (B) Phosphorylation levels of MAP kinases were quantified by densitometer and expressed as fold induction. Data are the mean ± S.E.M. of three independent experiments. **p*<0.05; compared with the control.

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-

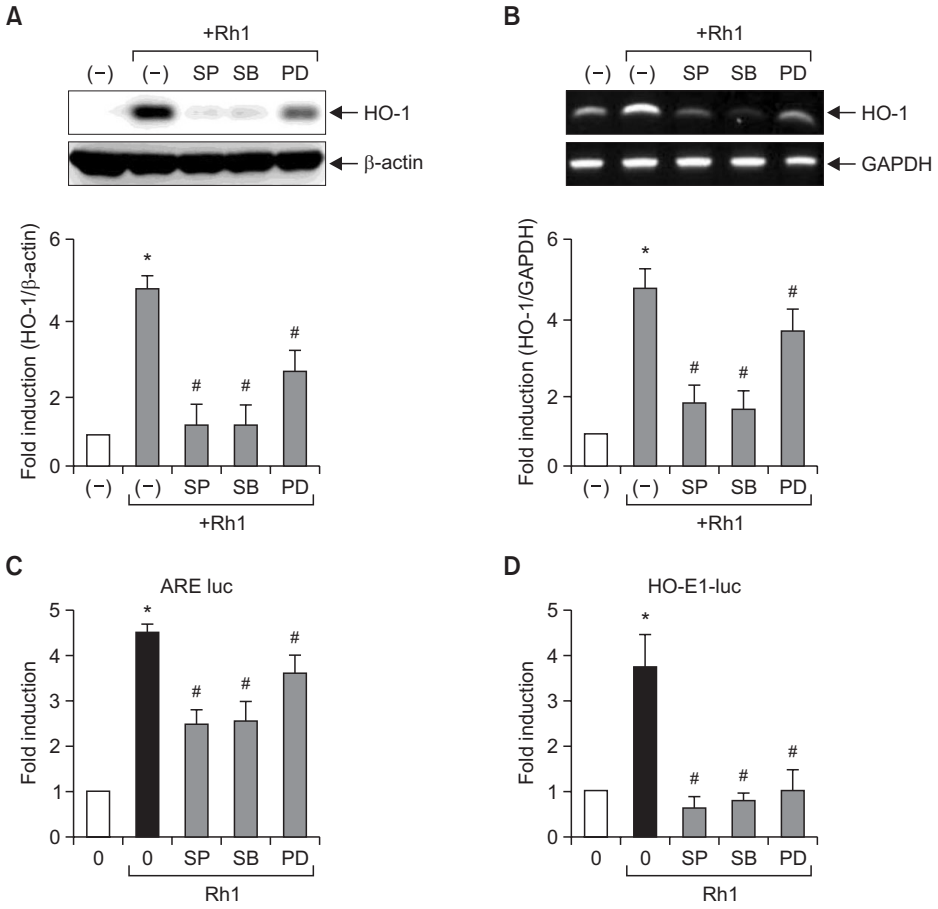

Fig. 5. MAPK signaling pathways are involved in HO-1 expression and ARE-mediated transcriptional activation in astrocytes. (A) Effects of MAPK inhibitors on HO-1 protein expression. Cells were treated with Rh1 (300 μM) in the presence of inhibitors for 12 h, and western blot analysis was performed. The blots are representative of three independent experiments. (B) Effects of MAPK inhibitors on HO-1 mRNA expression. Cells were treated with Rh1 (300 μM) in the presence of inhibitors for 12 h, and RT-PCR analysis was performed. The blots are representative of three independent experiments. Quantification data are shown at the bottom panel. (C, D) Primary astrocytes were transfected with ARE-luc (C) or HO-E1-luc (D), and treated with Rh1 (300 μM) in the absence or presence of MAPK inhibitors. After 24 h, cells were harvested and the luciferase assay was performed. Values correspond to the mean ± S.E.M. of three independent experiments. *p<0.05; compared with the control cells. $^{\#}p$<0.05; compared with the cells treated with Rh1. SP (20 μM of SP600125 [a JNK inhibitor]), SB (20 μM of SB203580 [a p38 MAPK inhibitor]), and PD (20 μM of PD98059 [an ERK1/2 inhibitor]).

ed cells, Western blot analysis was performed. As shown in Fig. 4, 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. 5A, 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 H_2O_2, 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 IFN-γ-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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