Lonchocarpine Increases Nrf2/ARE-Mediated Antioxidant Enzyme Expression by Modulating AMPK and MAPK Signaling in Brain Astrocytes

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Abstract

Lonchocarpine is a phenylpropanoid compound isolated from *Abrus precatorius* that has anti-bacterial, anti-inflammatory, antiproliferative, and antiepileptic activities. In the present study, we investigated the antioxidant effects of lonchocarpine in brain glial cells and analyzed its molecular mechanisms. We found that lonchocarpine suppressed reactive oxygen species (ROS) production and cell death in hydrogen peroxide-treated primary astrocytes. In addition, lonchocarpine increased the expression of antioxidant enzymes, such as heme oxygenase-1 (HO-1), NAD(P)H:quinone oxidoreductase 1 (NQO1), and manganese superoxide dismutase (MnSOD), which are all under the control of Nrf2/antioxidant response element (ARE) signaling. Further, mechanistic studies showed that lonchocarpine increases the nuclear translocation and DNA binding of Nrf2 to ARE as well as ARE-mediated transcriptional activities. Moreover, lonchocarpine increased the phosphorylation of AMP-activated protein kinase (AMPK) and three types of mitogen-activated protein kinases (MAPKs). By treating astrocytes with each signaling pathway-specific inhibitor, AMPK, c-jun N-terminal protein kinase (JNK), and p38 MAPK were identified to be involved in lonchocarpine-induced HO-1 expression and ARE-mediated transcriptional activities. Therefore, lonchocarpine may be a potential therapeutic agent for neurodegenerative diseases that are associated with oxidative stress.

Key Words: Lonchocarpine, Astrocytes, Antioxidant enzymes, AMPK, MAPK, Nrf2/ARE signaling

INTRODUCTION

Astrocytes are the most abundant cell types in the brain and provide structural, trophic, and metabolic support to neurons in addition to modulating synaptic activity (Sofroniew and Vinters, 2010; Finsterwald *et al*., 2015). In particular, astrocytes are enriched with antioxidant enzymes and maintain a homeostatic environment for neurons by controlling oxidative stress (Drukarch *et al*., 1998; Vargas and Johnson, 2009). Reactive oxygen species (ROS), such as hydrogen peroxide, superoxide, and highly reactive hydroxyl radicals, contribute to DNA/protein/tissue damage, inflammation, and neuronal apoptosis (de Vries *et al*., 2008). In particular, ROS production is known to be associated with the generation of neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, and multiple sclerosis (Barnham *et al*., 2004; de Vries *et al*., 2008). Therefore, controlling oxidative stress is an important therapeutic strategy for treating neurodegenerative diseases.

Lonchocarpine is a phenylpropanoid compound isolated from *Abrus precatorius*, which is a broadly distributed tropical medicinal plant (Gul *et al*., 2013). *Abrus precatorius* have been shown to have anti-inflammatory, anti-bacterial, antiproliferative, and antiepileptic activities (Georgewill and Georgewill, 2009; Premanand and Ganesh, 2010; Gul *et al*., 2013). The extract of *Abrus precatorius* produces an anti-inflammatory response following topical application to the ear of the rat after inducing inflammation by treatment of croton oil. The amount of reduction of the inflammatory response by the extract was similar to that by acetylsalicylic acid (Georgewill and Georgewill, 2009). Moreover, the petroleum ether extract of *Abrus precatorius* exhibits neuroprotection against hypoxic neurotoxicity. The extract recovered decreased levels of dopamine while reducing glutamate levels and acetylcholinesterase activity in hypoxia-induced rat brains (Premanand and Ganesh, 2010). Lonchocarpine is one of the major compo-
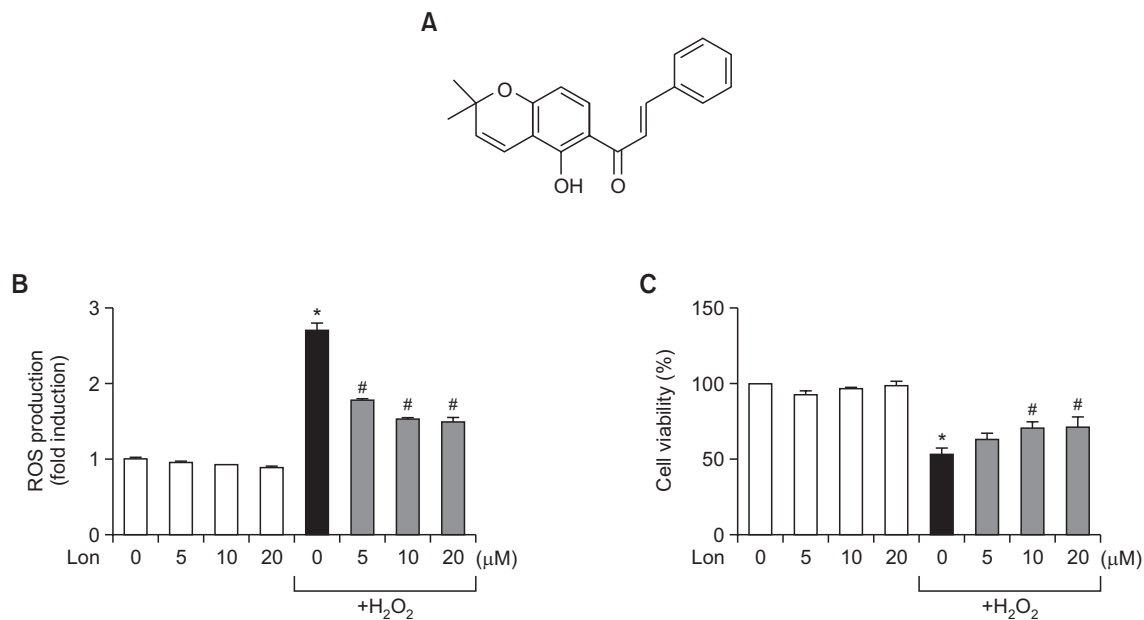
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A

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Fig. 1. Effects of lonchocarpine on ROS production in rat primary astrocytes. (A) Structure of lonchocarpine ((E)-1-(5-hydroxy-2,2-dimethyl-2H-chromen-6-yl)-3-phenylprop-2-en-1-one). (B) Rat primary astrocyte cells were treated with 5 to 20 μM lonchocarpine for 1 h, followed by treatment of H_2O_2 (50 μM) for 30 min. The intracellular ROS levels were then measured by the DCF-DA method. (C) Rat primary astrocyte viability was determined by the MTT assay after 24 h of H_2O_2 (50 μM) treatment. The data are expressed as the mean ± S.E.M. of three independent experiments. *$p<0.05$, significantly different from control samples; #$p<0.05$, significantly different from H_2O_2-treated samples.

nents of *Abrus precatorius*. Recently, the biological effects of lonchocarpine were reported in several papers. Lonchocarpine showed gastroprotective effects by inhibiting H^+,K^+-ATPase activity (Reyes-Chilpa *et al*., 2006). It also showed acute anti-inflammatory and anti-oedematogenic properties in a paw oedema model (Fontenele *et al*., 2009). In addition, lonchocarpine has anti-bacterial activity on *Staphylococcus aureus* (Lima *et al*, 2013). A recent study reported that lonchocarpine inhibited nitric oxide production in lipopolysaccharide-stimulated BV2 microglial cells (Li *et al*., 2015). However, the antioxidant effect of lonchocarpine in astrocytes has not been reported thus far.

Therefore, in the present study, we investigated the effects of lonchocarpine on ROS production and expression of antioxidant enzymes in primary cultured astrocytes. Lonchocarpine inhibited ROS production induced by hydrogen peroxide and suppressed astroglial cell death. In addition, lonchocarpine increased the expression of antioxidant enzymes by upregulating Nrf2/ARE signaling. Further, mechanistic studies showed that AMPK and MAPK pathways are involved in HO-1 expression by modulating Nrf2/ARE signaling. The data collectively suggest a therapeutic potential for lonchocarpine in various neurodegenerative diseases.

MATERIALS AND METHODS

Isolation of lonchocarpine from *Abrus precatorius*

The root of *Abrus precatorius* (3 kg) was extracted three times with 80% MeOH under reflux at boiling temperature for 3 hours, filtered, and concentrated to obtain a MeOH extract (600 g). The MeOH extract was suspended in hot water and then partitioned with EtOAc (2.0 L×3) and BuOH (2.0 L×3) to generate EtOAc- (80 g) and BuOH- (350 g) soluble fractions. The BuOH fraction was chromatographed using a silica-gel column (40×15 cm) and eluted with a gradient of chloroform and MeOH (100:1 to 5:1) and then chloroform, MeOH, and water (65:35:15 to 0:100:0) to generate 12 fractions (B1-B12). Fraction B4 was used to generate four subfractions (B4.1-4.4) using a silica-gel column (30×7 cm) eluted with a gradient of chloroform and EtOAc (20:1-5:1), then chloroform and acetone (7:1-3:1), and finally chloroform and MeOH (5:1-0:100). Compound 1 (1.2 g) was isolated from these subfractions. This compound was identified as lonchocarpine by instrumental analysis and compared to an authentic standard described in the literature (Lima *et al*., 2013). The purity of lonchocarpine was more than 95%. The structure of lonchocarpine is shown in Fig. 1A.

Reagents

Antibodies against antioxidant enzymes (HO-1, NQO1, and MnSOD) and oligonucleotides for electrophoretic mobility shift assays (EMSAs) were purchased from Santa Cruz (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Antibodies against MAPKs and phosphorylated forms of AMPK were purchased from Cell Signaling Technology (Danvers, MA, USA). All reagents used for RT-PCR were purchased from Promega (Madison, WI, USA). Compound C (an AMPK inhibitor) and MAPK inhibitors (SB203580, PD98059, SP600125) were purchased from Merck-Millipore (Billerica, MA, USA). All other chemicals were obtained from Sigma Chemical Corporation (St. Louis, MO, USA) unless stated otherwise.

Rat primary astrocyte culture

Primary astrocyte cultures were prepared from mixed glial cultures by modifying a previously published method (Park *et*
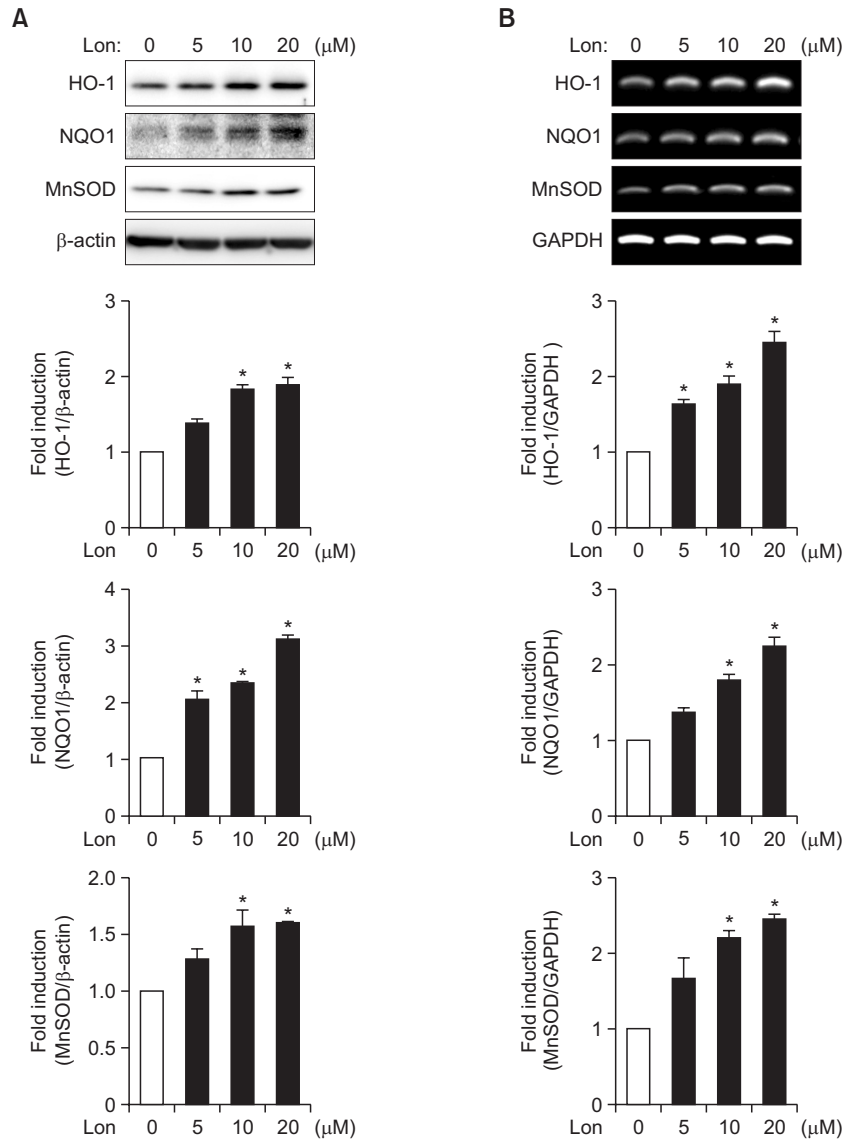
Fig. 2. Effect of lonchocarpine on anti-oxidant enzyme expression in rat primary astrocytes. (A) Cells were incubated with lonchocarpine for 16 h, after which protein expression of antioxidant enzymes was determined by Western blotting. (B) Cells were treated with various concentrations of lonchocarpine for 6 h and total RNA was isolated. The HO-1, NQO1, and MnSOD mRNA levels were determined by RT-PCR analysis. The data shown are representative of three independent experiments. Quantification data are shown at the bottom of each panel. *p<0.05, significantly different from control sample.

al., 2011). In brief, after cortices were dissected from 1-day-old rats, cells were dissociated by pipetting through pores of different sizes and resuspended in minimum essential medium (MEM) 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 days. After the primary culture reached confluence, the culture flasks were shaken at 280 rev/min for 16 h to remove microglia and oligodendrocytes. Astrocyte-enriched culture purity (>95%) were confirmed by staining with antibodies against the astrocyte-specific marker glial fibrillary acidic protein (GFAP).

Measurement of intracellular ROS levels

Intracellular accumulation of ROS was measured using a modification of a previously described method (Park et al., 2011). In brief, astrocytes were stimulated with hydrogen peroxide (H_2O_2) for 30 min and then stained with 50 μM dichlorodihydrofluorescein diacetate (H_2DCFDA) in phosphate-buffered saline (PBS) 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, Palo Alto, CA, USA).

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared from astrocytes as previously described (Lee et al., 2015). Double-stranded DNA

oligonucleotides containing the ARE consensus sequences (Santa Cruz) were end-labeled with [γ-32P]ATP. Thirty 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.*, 2015).

Western blot analysis

Cells were appropriately treated, and total cell lysates and nuclear extracts were prepared as described previously (Park *et al.*, 2009; Lee *et al.*, 2015). The proteins (20-50 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (GE Healthcare, Little Chalfont, 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), then incubated with primary antibodies (1:1000) that recognize HO-1, NQO1, MnSOD, Nrf2, β-actin, or lamin A. We also used antibodies that recognize the phospho-forms of AMPK or MAPKs. After thoroughly washing with TBST, horseradish peroxidase-conjugated secondary antibodies (1:2000 dilution in TBST; BioRad, Hercules, CA, USA) were applied, and the blots were developed using an enhanced chemiluminescence detection kit (Thermo Fisher Scientific, Waltham, MA, USA).

RT-PCR

Primary astrocytes were treated with lonchocarpine, and total RNA was isolated with TRI reagent (Ambion, Austin, TX, USA). For RT-PCR, total RNA (1 μg) was reverse transcribed in a reaction mixture containing 1 U RNase inhibitor, 500 ng random primers, 3 mM MgCl2, 0.5 mM dNTP, 1× RT buffer, 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 follows: 5'-TGTCACCCTGTGCTTGACCT-3' (sense) and 5'-GCCATGAAGGAGGCTGCT GT-3' (antisense) for HO-1; 5'-ATCACCAGGTCTGCAGCTTC-3' (sense) and 5'-GAACCTTGGACTCCCACAGA-3' (antisense) for NQO1; 5'-GGCCAAGGGAG ATGTTACAA-3' (sense) and 5'-GAAC-CTTGGACTCCCACAGA-3' (antisense) for MnSOD; 5'-GTG-CTGAGTATGTCGTGGAGTC-3' (sense) and 5'-ACAGTCTTC-TG AGTGGCAGTCA-3' (antisense) for GAPDH.

Transient transfection and luciferase assay

Rat primary astrocytes were plated on a 12-well plate at a density of 2.5×10^5 cells/ml, and transfected with 0.5 μg of plasmid DNA using Convoy™ Platinum transfection reagent (ACTGene, Inc., Kendall Park, NJ, USA). To determine the effect of lonchocarpine on ARE promoter activity, cells were treated with lonchocarpine and incubated for 16 h prior to harvesting cells, after which a luciferase assay was performed as previously described (Park *et al.*, 2011).

Statistical analysis

Unless otherwise stated, all of the experiments were performed with triplicate samples and repeated at least three times. The data are presented as the mean ± S.E.M., and statistical comparisons between groups were performed using one-way analysis of variance followed by the Newman-Keuls test. A *p*-value of less than 0.05 was considered to be statistically significant.
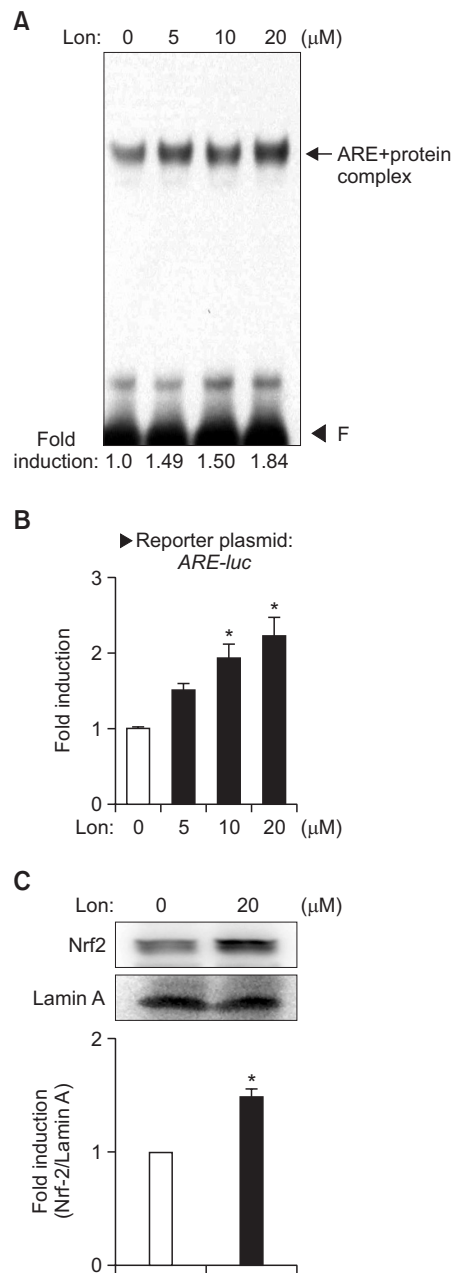
Fig. 3. Effect of lonchocarpine on nuclear protein binding to ARE and ARE-mediated transcriptional activities. The nuclear extracts were isolated from cells treated with 5 to 20 μM lonchocarpine for 3 h. (A) The extracts were incubated with a ^{32}P-labeled ARE probe. The arrow indicates the ARE-nuclear protein complex. 'F' indicates free probe. (B) Primary astrocytes were transfected with ARE-luc reporter plasmids and treated with lonchocarpine. After 16 h, cells were harvested and luciferase assays were performed. (C) The nuclear extracts were assessed by Western blot using antibodies against Nrf2. The data shown are representative of three independent experiments. Values correspond to the mean ± S.E.M. of three independent experiments. *p<0.05, compared with control cells.
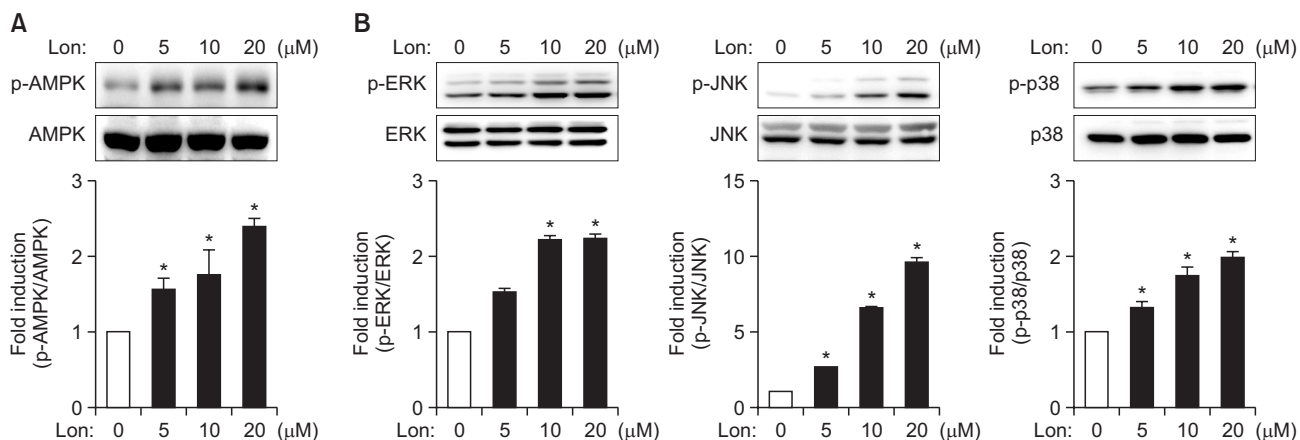
Fig. 4. Effects of lonchocarpine on AMPK and MAPK phosphorylation. Cell extracts were prepared from astrocytes treated with lonchocarpine for 1 h and they were subjected to immunoblot analysis using antibodies against phospho- or total forms of AMPK (A) or three types of MAP kinases (ERK1/2, JNK, p38 MAPK) (B). The blots shown are representative of three independent experiments. Phosphorylation levels of AMPK and MAP kinases were quantified by densitometry and expressed as fold inductions (bottom panel). *$p<0.05$, compared with control cells.

RESULTS

Lonchocarpine inhibited ROS production in H_2O_2-treated primary astrocytes

To determine the antioxidant capacity of lonchocarpine, we examined the effects of lonchocarpine on ROS production in H_2O_2-treated astrocytes. We found that lonchocarpine significantly inhibited H_2O_2-induced ROS production (Fig. 1B). In addition, lonchocarpine attenuated astroglial cell death induced by H_2O_2 (Fig. 1C). The data suggest that lonchocarpine may have cytoprotective effects by inhibiting ROS production.

Lonchocarpine increased the expression of HO-1, NQO1, and MnSOD in astrocytes

Next, we examined whether lonchocarpine induces the expression of antioxidant enzymes that play a pivotal role in cellular defense mechanisms against oxidative stress (Sypin, 2008; Vargas and Johnson, 2009). Using western blot analysis we found that lonchocarpine increased the protein expression of antioxidant enzymes such as HO-1, NQO1, and MnSOD in rat primary astrocytes (Fig. 2A). In addition, lonchocarpine upregulated the expression of those enzymes at the mRNA level as shown by RT-PCR analysis (Fig. 2B). The data suggest that lonchocarpine regulates antioxidant enzyme genes at a transcriptional level.

Lonchocarpine increased nuclear protein binding to ARE and ARE-mediated transcriptional activities in astrocytes

To further investigate the molecular mechanisms underlying upregulation of antioxidant enzymes by lonchocarpine, we examined the effects of lonchocarpine on Nrf2/ARE signaling, which plays a key role in the gene expression of antioxidant enzymes such as HO-1, NQO1, and MnSOD (Vargas and Johnson, 2009). In particular, Nrf2 is known to bind to the ARE sequence on the promoter of antioxidant enzyme genes and regulate their expression (Jaiswal, 2004). In the present study, we found that lonchocarpine increased nuclear protein binding to ARE (Fig. 3A). Transient transfection analysis showed that lonchocarpine also increased ARE-mediated transcrip-

tional activities in astrocytes (Fig. 3B). In support of this, lonchocarpine increased translocation of Nrf2 into the nuclei of astrocytes (Fig. 3C). The data suggest that lonchocarpine increases antioxidant enzyme gene expression by enhancing Nrf2/ARE signaling.

AMPK and MAPK signaling are involved in antioxidant enzyme expression by modulating the Nrf2/ARE axis in lonchocarpine-treated astrocytes

Previous studies have reported that phase II antioxidant enzyme expression is under the control of AMPK, PI3K, MAPK, or protein kinase C signaling pathways in various cell types (Jaiswal, 2004; Niture *et al*, 2010). Here, we performed western blot analysis to determine the signaling pathways involved in antioxidant enzyme gene expression in lonchocarpine-treated cells. As shown in Fig. 4, lonchocarpine increased the phosphorylation of AMPK and three types of MAPKs. To determine whether AMPK and MAPKs mediate the expression of HO-1 as a representative of antioxidant enzymes, cells were treated with each signaling pathway-specific inhibitor before stimulation with lonchocarpine. We found that treatment with AMPK or a JNK inhibitor (compound C, SP600125) suppressed HO-1 expression in lonchocarpine-treated astrocytes (Fig. 5A). However, an ERK inhibitor (PD98059) did not affect the HO-1 expression. Intriguingly, treatment with a p38 inhibitor (SB203580) increased HO-1 expression. When we examined the effects of these inhibitors on Nrf2/ARE, similar patterns of changes were observed; Nrf2 DNA-binding and ARE reporter gene activities induced by lonchocarpine were decreased by compound C and SP600125, increased by SB203580, and unaltered by PD98059 (Fig. 5B, 5C). We observed that the inhibitor-only condition did not show any change in the basal level of HO-1 expression or ARE-mediated transcription (data not shown & Fig. 5B). The data suggest that lonchocarpine-induced HO-1 expression is positively regulated by JNK and AMPK, while negatively regulated by p38 MAPK via Nrf2/ARE signaling.
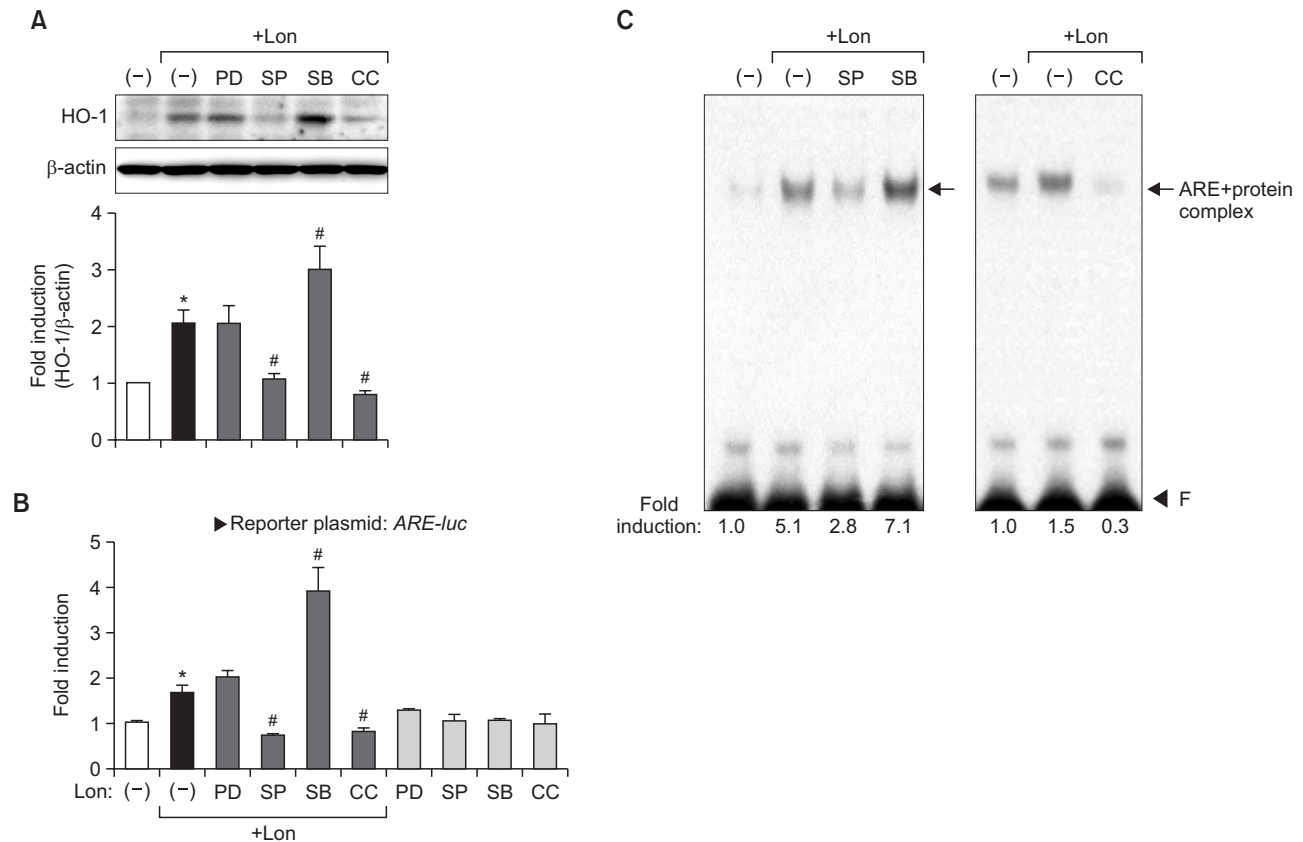
Fig. 5. Effects of AMPK or MAPK inhibitors on ARE-mediated HO-1 expression in lonchocarpine-treated astrocytes. (A) Effects of AMPK and MAPK inhibitors on HO-1 protein expression. Cells were treated with the inhibitors, followed by treatment with lonchocarpine (20 μM) for 16 h, after which western blot analyses were performed. The blots shown are representative of three independent experiments. Quantification data are shown at the bottom panel. (B) Effects of AMPK and MAPK inhibitors on ARE-mediated transcriptional activity. Primary astrocytes were transfected with ARE-luc and treated with lonchocarpine (20 μM) in the presence or absence of inhibitors. (C) Effect of inhibitors on nuclear protein binding to ARE. CC; compound C (AMPK inhibitor, 10 μM), SP; SP600125 (JNK inhibitor, 20 μM), SB; SB203580 (p38 inhibitor, 20 μM), PD; PD98059 (ERK inhibitor, 20 μM). *$p < 0.05$, compared with control samples. #$p < 0.05$, compared with lonchocarpine-treated samples.

DISCUSSION

In the present study, we demonstrated that lonchocarpine inhibited ROS production in H_2O_2-treated rat primary astrocytes. Lonchocarpine increased anti-oxidant enzymes such as HO-1, NQO1, and MnSOD by enhancing Nrf2/ARE signaling. In addition, AMPK, JNK and p38 signaling pathways were identified to be involved in HO-1 expression by modulating Nrf2 binding and ARE-mediated transcriptional activities in lonchocarpine-treated astrocyte cells.

Several studies have reported that the Nrf2/ARE signaling pathway is a promising therapeutic target for neuroinflammation and brain disorders as it plays a key role as a regulator of cellular redox status (Syapin, 2008; Johnson and Johnson, 2015). Nrf2/ARE signaling governs phase II antioxidant gene expression such as HO-1 and NQO1, and Nrf2 activation has shown neuroprotective effects in animal models of cerebral ischemia, Parkinson's disease, Alzheimer's disease, and amyotrophic lateral sclerosis (Vargas *et al*., 2008; Calkins *et al*., 2009; Johnson and Johnson, 2015). Thus, the upregulation of Nrf2/ARE signaling by lonchocarpine may contribute to neuroprotection against oxidative stress.

Recently, AMPK activation has been considered a potential therapeutic target against oxidative stress-induced neuronal damage (de Vries *et al*., 2008; Carling *et al*., 2012). AMPK is a serine/threonine kinase that plays a pivotal role in redox signaling and cellular energy homeostasis (Zang *et al*., 2004; Zimmermann *et al*., 2015). It has been shown that AMPK has neuroprotective effects by reducing oxidative damage and recovering normal brain function in cerebral ischemia (Ronnett *et al*., 2009). In addition, AMPK protects against cholesterol-induced and amyloid β-accumulated neurotoxicity (Martinez de Morentin *et al*., 2010; Vingtdeux *et al*., 2010). Several papers have demonstrated the interaction of AMPK and Nrf2 signaling. AMPK has been shown to induce HO-1 expression via Nrf2/ARE signaling in mouse embryonic fibroblasts and endothelial cells (Liu *et al*., 2011; Zimmermann *et al*., 2015). AMPK also plays an important role in the anti-inflammatory effects of berberine by modulating Nrf2 in macrophages (Mo *et al*., 2014). In accordance with these results, our present study showed that AMPK plays a critical role in HO-1 expression by modulating Nrf2 signaling in lonchocarpine-treated astrocyte cells.

MAPK signaling pathways are also related to the upregula-

tion of phase II antioxidant enzymes (Jaiswal, 2004; Niture et al., 2010). For example, JNK induces Nrf2 binding to ARE and improves endogenous antioxidant responses in murine macrophages (Xu et al., 2006; Vari et al., 2011). The ERK and p38 MAPK have been implicated in HO-1 activation by arsenite in hepatocytes (Elbirt et al., 1998; Ryter et al., 2002). In addition, many studies implied a combined role of more than one MAPK pathway in gaining optimal gene expression (Chen and Maines, 2000; Ryter et al., 2006). Furthermore, the studies indicate that there is considerable variation in the type of MAPK(s) involved in HO-1 activation in an inducer-specific, cell type-specific and species-specific manner (Ryter et al., 2006). In the present study, we found that lonchocarpine induced phosphorylation of three types of MAPKs. By treating astrocytes with specific inhibitors, we found that JNK is responsible for HO-1 upregulation via Nrf2/ARE. In contrast, p38 MAPK appears to be involved in HO-1 expression in the opposite manner, and ERK is not involved in this regulation. Therefore, the coordinated regulation by AMPK, MAPKs, and other signaling pathways may result in the final outcome of upregulation of antioxidant enzyme expression in lonchocarpine-treated astrocytes. Further study is necessary to clarify the mechanisms underlying this differential regulation of HO-1 by MAPKs and other signaling pathways.

In conclusion, the present study has demonstrated that lonchocarpine has antioxidant effects and that it upregulates antioxidant enzyme expression by modulating AMPK, MAPKs, and Nrf2 signaling pathways. The antioxidant effects of lonchocarpine may have potential therapeutic utility for various neurodegenerative diseases.

ACKNOWLEDGMENTS

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