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Cytoprotective effects of lindenenyl acetate isolated from *Lindera strychnifolia* on mouse hippocampal HT22 cells

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ABSTRACT

Oxidative injury contributes to neuronal degeneration in many central nervous system (CNS) diseases, such as Parkinson's disease, Alzheimer's disease, epilepsy and ischemia. Inducible heme oxygenase (HO)-1 acts against oxidants that are thought to play a role in the pathogenesis of these diseases. Lindenenyl acetate, isolated by bioassay-guided fractionation of the MeOH extract of the roots of *Lindera strychnifolia*, showed potent neuroprotective effects on glutamate-induced neurotoxicity by inducing the expression of HO-1 and increasing the activity of HO in mouse hippocampal HT22 cells. Furthermore, lindenenyl acetate caused the nuclear accumulation of nuclear factor-E2-related factor 2 (Nrf2) and increased the promoter activity of antioxidant response elements (ARE) in mouse hippocampal HT22 cells. In addition, we found that treatment of the cells with extracellular signal-regulated kinase (ERK) inhibitor (U0126) reduced lindenenyl acetate-induced HO-1 expression. Lindenenyl acetate also increased ERK phosphorylation. These results suggest that lindenenyl acetate increases cellular resistance to glutamate-induced oxidative injury in mouse hippocampal HT22 cells, presumably through the ERK pathway-Nrf2/ARE-dependent HO-1 expression.

1. Introduction

Oxidative stress, or the accumulation of reactive oxygen species, has been implicated in the pathogenesis of neuronal degenerative diseases such as Alzheimer's disease, Parkinson's disease and stroke, caused by leading neuronal cellular death and dysfunction (Coyle and Puttfarcken, 1993; Satoh et al., 1999, 2006; Satoh and Lipton, 2007). Glutamate is the main excitatory neurotransmitter in the central nervous system (CNS). Glutamate toxicity causes neuronal cell loss associated with acute insults and chronic neurodegenerative disease (Siesjö, 1981; Greenamyre et al., 1985). Glutamate toxicity also has been shown to induce neuronal cell death through both receptor-initiated excitotoxicity and non-receptor-mediated oxidative stress (Choi, 1988; Lipton, 2007). Moreover, HT22 cells have been used as a useful in vitro model for studying the mechanism of oxidative glutamate toxicity (Maher and Davis, 1996). Because immortalized neuronal HT22 cells, originating from mouse hippocampus, lack functional ionotropic glutamate receptors, thus excluding excitotoxicity as a cause for glutamate triggered cell death (Rössler et al., 2004; Jeong et al., 2007). Especially, the high concentrations of extracellular glutamate inhibited the uptake of cystine into the cells via the cystine/glutamate antiporter system, which caused the progressive depletion of glutathione, the major intracellular antioxidant in vitro model (Rössler et al., 2004; Breyer et al., 2007).

Heme oxygenase (HO) enzymes are important components of the cellular antioxidant system. The products of the heme oxygenase reaction, which include free ferrous iron, carbon monoxide, and biliverdin/bilirubin, have a number of potentially protective effects against oxidative stress (Morse and Choi, 2002; Lee et al., 2006a; Choi et al., 2002). HO consists of three isozymes: HO-1, HO-2 and HO-3. Although HO-2 and HO-3 are constitutively expressed, HO-1 is inducible in many cell types, such as neuronal cells (Schipper, 2004; Satoh et al., 2003). The expression of HO-1 also has cytoprotective effects in glutamate-induced oxidative cytotoxicity in HT22 cells (Satoh et al., 2003; Rössler et al., 2004). The induction of HO-1 is primarily regulated at the transcriptional level, and its induction by various inducers is related to the nuclear transcription factor-E2-related factor 2 (Nrf2) (Itoh et al., 1997). Nrf-2 is a basic leucine zipper transcription factor that resides in the cytoplasm bound to its inhibitor protein, Keap 1, and translocates to the nucleus after stimulation. It then binds to the antioxidant response element (ARE) sequences in the promoter regions of specific genes (Lee et al., 2006b; Qiang et al., 2004; Kim et al., 2007). Nrf2 has been known to induce the expression of antioxidant stress proteins such as HO-1 and glutathione (GSH) (Ishii et al., 2000; Lim et al., 2007).

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Mitogen-activated protein kinase (MAPK) is one of the most common signaling pathways that serve to coordinate the cellular response to a variety of extracellular stimuli. There are three members of MAPK subfamilies, including extracellular signal-regulated kinases (ERK1/2), p38 kinase, and c-Jun N-terminal kinase (JNK) (Choi et al., 2005; Satoh et al., 2000; Oh et al., 2006; Stanciu et al., 2000). The MAPK is activated in response to oxidative stress and various other stressors, and the MAPK activation also modulates several gene and protein expression, such as that of HO-1 (Stanciu et al., 2000; Iles et al., 2005).

Lindera strychnifolia Vill. (Lauraceae) is widely distributed in Japan and the People's Republic of China, and its roots, Linderae Radix, are used as a traditional medicine for treating kidney deficiencies such as pollakisuria or urinary incontinence (Hsu et al., 1986; Bensk and Gamble, 1986). In addition, *L. strychnifolia* extracts exert antiviral (Zheng, 1990), antioxidant (Noda and Mori, 2007), and antidiabetic activity (Ohno et al., 2005). Recent studies reported that *L. strychnifolia* extract demonstrated an inhibitory effect on prolyl endopeptidase (Kobayashi et al., 2002) and it also exerted a potentially protective effect against post-ischemic myocardial dysfunction (Wang et al., 2004). Previous phytochemical studies of *L. strychnifolia* have reported the isolation of linderol, linderane (Tori et al., 1975), lindesterene, linderene acetate (Takeda et al., 1964), isolinderoxide (Takeda et al., 1967) and other some sesquiterpenes, alkaloids (Kouno et al., 2001; Ishii et al., 1968) and tannins (Kobayashi et al., 2002).

Since the induction of HO-1 by several phytochemicals isolated from specific medicinal herbs have been widely recognized as an effective neuronal cellular strategy to counteract a variety of stressful stimuli (Jeong et al., 2008; Chen et al., 2005; Choi et al., 2002), HO-1 expression by pharmacological modulators may represent a useful target for therapeutic intervention. HO-1 has been proposed to play an important cellular defense role against oxidant injury. Therefore, we demonstrated that lindenenyl acetate, isolated from the root of *L. strychnifolia*, increased cellular resistance to oxidative injury caused by glutamate-induced cytotoxicity in mouse hippocampal HT22 cells, through Nrf2/ARE-dependent HO-1 expression, via activation of the ERK pathway.

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. Tin protoporphyrin IX (SnPP IX), an inhibitor of HO activity, was obtained from Porphyrin Products. All other chemicals were obtained from Sigma Chemical Co, unless indicated otherwise. Mouse hippocampal HT22 cells were received from Dr. Inhee-Mook (Seoul National University, Korea). The cells were maintained at 5×10^6 cells/dish in 100 mm dishes in DMEM medium supplemented with 10% heat-inactivated FBS, penicillin G (100 units/ml), streptomycin (100 mg/ml), and L-glutamine (2 mM) and incubated at 37 °C in a humidified atmosphere containing 5% CO_2 and 95% air.

2.2. Instruments

NMR spectra were recorded using a JEOL Eclipse-500 MHz spectrometer (500 MHz for ^{1}H, 125 MHz for ^{13}C), and chemical shifts are quoted vs. tetramethylsilane. Column chromatography was performed on silica gel 60 (70–230 mesh, Merck) and YMC gel (YMC Co. Ltd., Japan). In TLC, silica gel F_{254} plate (0.2 mm, Merck) and RP-18 F_{254s} plates (0.2 mm, Merck) were used. Spots were detected under UV light or after spraying with 10% H_2SO_4 reagent, flowed by heating.

2.3. Extraction and isolation

Dried roots of *L. strychnifolia* were purchased from the University Oriental herbal drugstore, Iksan, Korea, in December 2006, and a voucher specimen (No. WP 06-420) was deposited at the Herbarium of the College of Pharmacy, Wonkwang University (Korea). Pulverized *L. strychnifolia* roots (1.5 kg) were extracted with MeOH (2 l×2) for 3 h under reflux and the extract was concentrated in vacuo to obtain a MeOH extract (93.3 g). The extract was suspended in H_2O (1 l), and the resulting H_2O layer was partitioned with *n*-hexane (1 l×2), EtOAc (1 l×2), *n*-BuOH (1 l×2). The *n*-hexane-soluble fraction (14 g) was chromatographed on a silica gel column and eluted with hexane: EtOAc (15:1→4:1) to obtain four fractions (Fr. LH-1 ~4). Fr. LH-2 (3.37 g) was chromatographed using the YMC gel column with 85% MeOH to obtain four fractions (Fr. LH-2-1 ~2–4). Fr. LH-2-2 (1.8 g) was chromatographed on a silica gel column with *n*-hexane:EtOAc (30:1) to give lindenenyl acetate (1.27 g).

Lindenenyl acetate — yellow oil; ^{13}C NMR ($CDCl_3$, 125 Hz) δ: 171.3 ($\underline{C}OCH_3$), 153.8 (C-8), 149.0 (C-4), 138.8 (C-12), 119.3 (C-11), 117.6 (C-7), 107.9 (C-15), 66.0 (C-6), 64.8 (C-5), 41.6 (C-10), 38.3 (C-9), 27.2 (C-1), 23.1 (C-3), 18.4 (C-14), 16.8 (C-2), 8.8 (C-13) (Kazuo et al., 1975).

2.4. Cell viability assay

For determination of cell viability, cells (2×10^4 cells/well in 96-well plates) were incubated with 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) at the final concentration of 0.5 mg/ml for 4 h, and the formazan formed was dissolved in acidic 2-propanol; optical density was measured at 590 nm using a microplate reader (Bio-rad, Hercules, CA). The optical density of formazan formed in control (untreated) cells was taken as 100% viability.

2.5. Preparation of nuclear and cytosolic fraction

Cells were homogenized (1:20, w:v) in PER-Mammalian Protein Extraction buffer (Pierce Biotechnology, Rockford, I L) containing freshly-added protease inhibitor cocktail I (EMD Biosciences, San

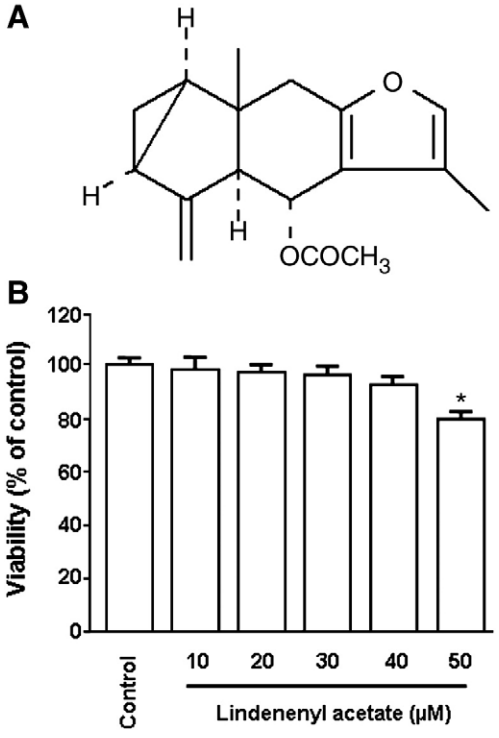

Fig. 1. The structure of lindenenyl acetate (A) and effects of lindenenyl acetate on cell viability; (B) HT22 cells were incubated for 12 h with various concentrations of lindenenyl acetate (10–50 μM). Cell viability was determined as described in the Materials and methods section. Each bar represents the mean ± S.D. of three independent experiments. *P<0.05 vs. control.

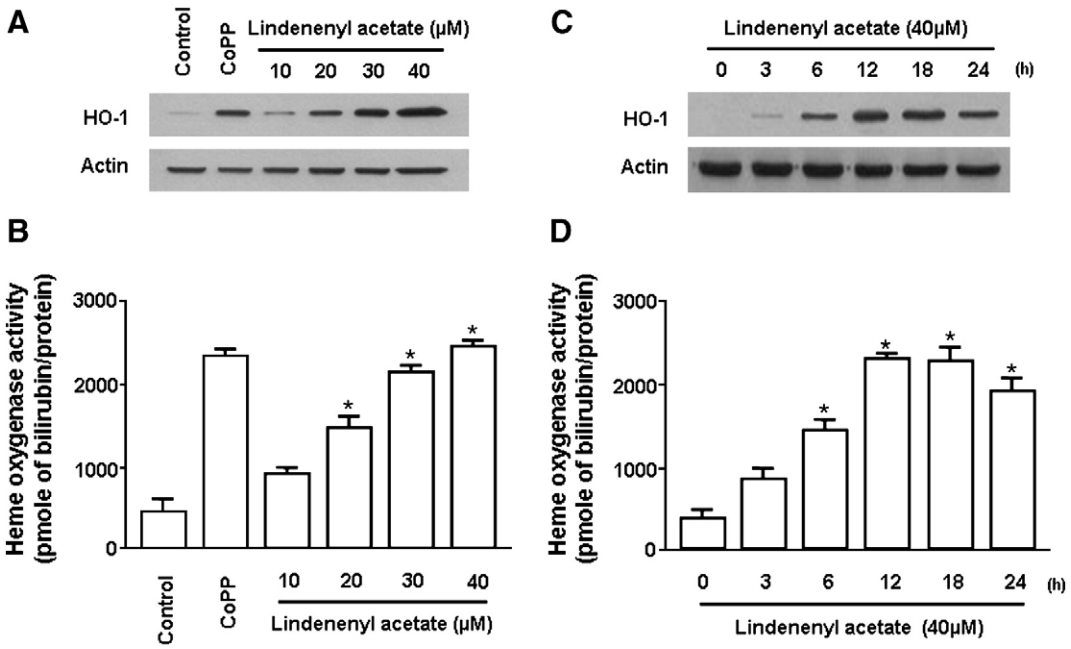

Fig. 2. Effects of lindenenyl acetate on HO-1 expression and HO activity in HT22 cells. (A) Cells were incubated for 12 h with lindenenyl acetate. (B) Cells were incubated for indicated periods with 40 μM of lindenenyl acetate. Expression of HO-1 was determined by Western blot analysis, and representative blots of three independent experiments are shown. (C) HO activity was determined via bilirubin formation at 12 h after treatment with various concentrations of lindenenyl acetate. (D) HT22 cells were treated with 40 μM of lindenenyl acetate, and HO activity was measured at the indicated time points. Each bar represents the mean ± S.D. of three independent experiments. *$P<0.05$ vs. control. CoPP (20 μM) was used as the positive control.

Diego, CA) and 1 mM phenylmethylsulfonyl fluoride (PMSF). The cytosolic fraction of the cell was prepared by centrifugation at 15,000 ×*g* for 10 min at 4 °C. Nuclear and cytoplasmic extracts of HT22 cells were prepared, using NE-PER nuclear and cytoplasmic extraction reagents (Pierce Biotechnology). After treatment, HT22 cells (3×10^6 cells/3 ml in 60 mm dish) were collected and washed with phosphate-buffered saline (PBS). After centrifugation, cell lysis was performed at 4 °C by vigorous shaking for 15 min in RIPA buffer [150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris–HCl (pH 7.4), 50 mM glycerophosphate, 20 mM NaF, 20 mM ethylene glycol tetraacetic acid (EGTA), 1 mM dithiothreitol (DTT), 1 mM Na_3VO_4, and protease inhibitors]. After centrifugation at 14,800 ×*g* for 15 min, the supernatant was separated and stored at −70 °C until further use. Protein content was determined using the bicinchoninic acid (BCA) protein assay kit.

2.6. Plasmids, transfections and luciferase assays

To construct the ARE-Luciferase vector, tandem repeats of double-stranded oligonucleotides spanning the Nrf2 binding site 5′-TGACT-CAGCA-3′ were introduced into the restriction sites of the pGL2 promoter plasmid (Madison, WI). All transfection experiments were performed using Lipofectamine reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. For luciferase assays, the cell lysate was first mixed with the luciferase substrate solution (Promega), and luciferase activity was measured using a luminometer. For each experiment, luciferase activity was determined in triplicate and normalized for each sample using β-galactosidase activity.

2.7. Western blot analysis

Western blot analysis was performed by lysing cells in 20 mM Tris–HCl buffer (pH 7.4), containing a protease inhibitor mixture (0.1 mM PMSF, 5 mg/ml aprotinin, 5 mg/ml pepstatin A, and 1 mg/ml chymostatin). Protein concentration was determined with the Lowry protein assay kit (P5625; Sigma). An equal amount of protein for each

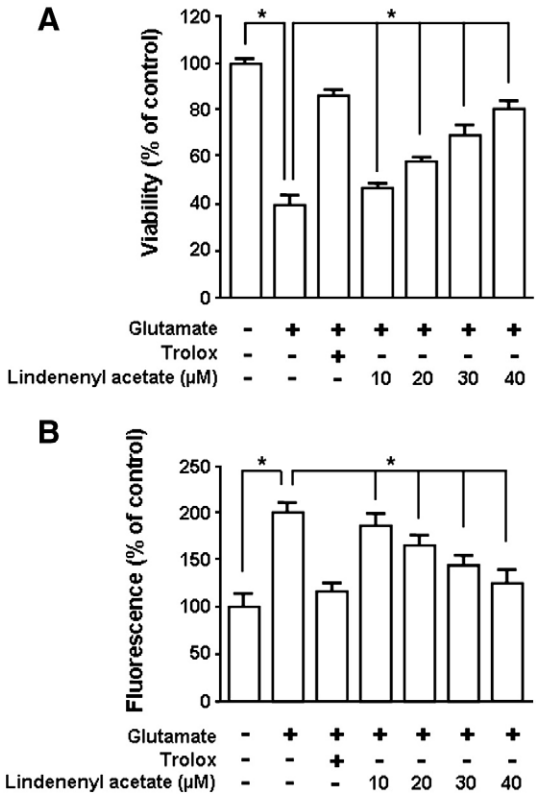

Fig. 3. Effects of lindenenyl acetate on glutamate-induced oxidative neurotoxicity and inhibition of reactive oxygen species generation. (A) Cells were treated with lindenenyl acetate and then incubated for 12 h with glutamate (5 mM). (B) Exposure of HT22 cells to 5 mM glutamate for 12 h increased reactive oxygen species production. Lindenenyl acetate effectively inhibited glutamate-mediated reactive oxygen species production. Each bar represents the mean ± S.D. of three independent experiments, *$P<0.05$. Trolox (50 μM) was used as the positive control.

sample was separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred onto a Hybond enhanced chemiluminescence (ECL) nitrocellulose membrane (Biorad). The membrane was blocked with 5% skim milk and sequentially incubated with primary antibody (Santa Cruz Biotechnology) and horseradish peroxidase-conjugated secondary antibody, followed by ECL detection (Amersham).

2.8. HO activity assay

HO enzyme activity was measured using a method previously described (Tenhunen et al., 1970). Briefly, microsomes from harvested cells were added to a reaction mixture containing nicotinamide adenine dinucleotide phosphate, biliverdin reductase from rat liver cytosol, and the substrate hemin. The reaction was conducted at 37 °C in the dark for 1 h, terminated by the addition of 1 ml of chloroform, and extracted bilirubin was calculated by the difference in absorbance between 464 and 530 nm.

2.9. Reactive oxygen species measurement

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

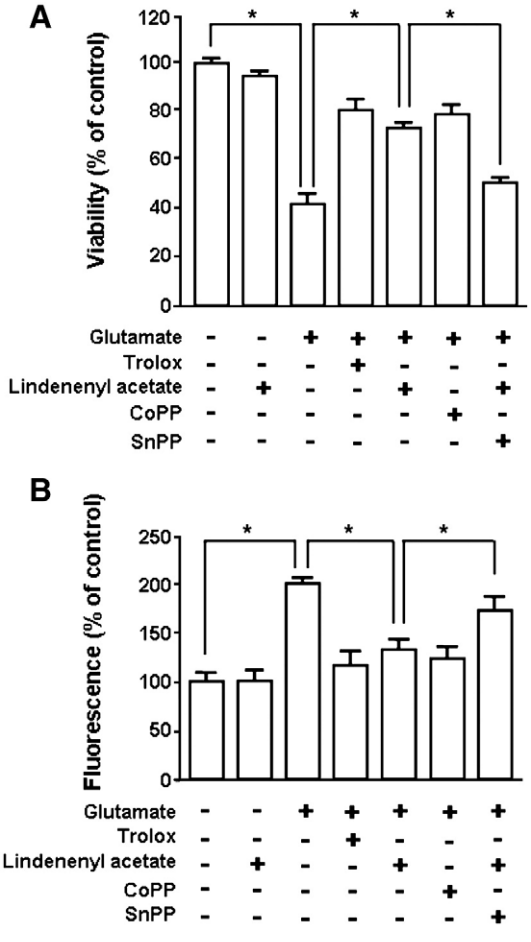

Fig. 4. Effects of lindenenyl acetate-induced HO-1 on glutamate-induced oxidative neurotoxicity and reactive oxygen species generation. (A) Cells were treated with 40 μM of lindenenyl acetate or 20 μM CoPP in the presence or absence of 50 μM SnPP and then exposed to glutamate (5 mM) for 12 h. (B) Exposure of HT22 cells to 5 mM glutamate for 12 h increased reactive oxygen species production. Lindenenyl acetate-induced HO-1 effectively inhibited reactive oxygen species production. Each bar represents the mean ± S.D. of three independent experiments, *$P<0.05$. Trolox and CoPP were used as the positive control.

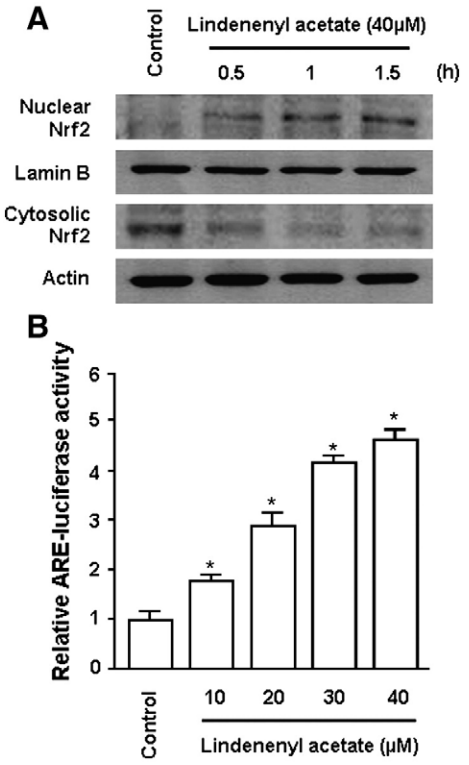

Fig. 5. Effects of lindenenyl acetate on Nrf-2 nuclear translocation and ARE activation in HT22 cells. (A) Cells were treated with 40 μM lindenenyl acetate for 0.5, 1, and 1.5 h. The nuclei were fractionated from the cytosol using PER-Mammalian Protein Extraction buffer as described in Materials and methods. Nrf-2 protein was detected by Western blot analysis, and representative blots of three independent experiments are shown. (B) Quiescent cells transiently transfected with ARE-luciferase or control vector were incubated for 1 h with indicated concentrations of lindenenyl acetate in the presence of 5% FBS. Cell lysates were assayed for luciferase activity as the fold induction by normalizing the transfection efficiency and dividing values of each experiment relative to the control. *$P<0.05$ vs. control.

2.10. Statistical analysis

Data are expressed as mean ± S.D. of at least three independent experiments. To compare three or more groups, one-way analysis of variance (ANOVA) followed by Newman–Keuls post hoc test was used. Statistical analysis was performed with GraphPad Prism software, version 3.03 (GraphPad Software Inc, San Diego, CA).

3. Result

3.1. Effects of lindenenyl acetate on cell viability, HO-1 expression and HO activity

To investigate the cytotoxic potential of lindenenyl acetate (Fig. 1A), its effect on viability of mouse hippocampal HT22 cells was first measured. No cytotoxic effects of lindenenyl acetate were reported up to a concentration of 40 μM, using the MTT assay. However, higher amounts reduced the viability of the HT22 cells (Fig. 1B). At non-cytotoxic concentrations (10–40 μM), we examined

whether lindenenyl acetate affected HO-1 protein expression and HO activity by treating the HT22 cells with this agent for 12 h (Fig. 2A and C). Lindenenyl acetate dose-dependently increased HO-1 expression (Fig. 2A) and HO activity (Fig. 2C). The HO-1 inducer CoPP, as a positive control, increased HO-1 expression and showed significant HO activity at 20 μM (Fig. 2A and C). The induction of HO-1 by lindenenyl acetate reached a peak at 40 μM. At a concentration of 40 μM, HO-1 induction was evident after 6 h, and reached a maximum after 18 h of treatment (Fig. 2B). HO activity also showed maximal activity at 18 h after treatment (Fig. 2D). This enhanced HO activity directly correlated with HO-1 protein level.

3.2. Effects of lindenenyl acetate on cell viability and in DCFDA fluorescence via HO-1 expression

Glutamate treatment increased HT22 cell death to 61% compared to the untreated cells. Lindenenyl acetate (10, 20, 30 and 40 μM) increased cytoprotection dose-dependently (Fig. 3A). Glutamate also doubled reactive oxygen species production, and lindenenyl acetate effectively suppressed this induction (Fig. 3B). Trolox (Sigma), well-known for its anti-oxidative efficiency, was used as a positive control, and showed a significantly cytoprotective effect and reactive oxygen species scavenging activity at a concentration of 50 μM (Fig. 3A and B). HO-1 also exerted cytoprotective effects in glutamate-induced oxidative cytotoxicity in HT22 cells (Coyle and Puttfarcken, 1993; Jeong et al., 2008). Thus, we tested whether lindenenyl acetate-induced HO-1 expression mediated this cytoprotective effect. HT22 cells were co-treated with 40 μM of lindenenyl acetate for 12 h in the absence or presence of SnPP, an inhibitor of HO activity (Fig. 4A). The HO-1 inducer CoPP showed comparable protection to lindenenyl acetate. The HO-1 expression by lindenenyl acetate was also required for suppressing glutamate-induced reactive oxygen species generation (Fig. 4B).

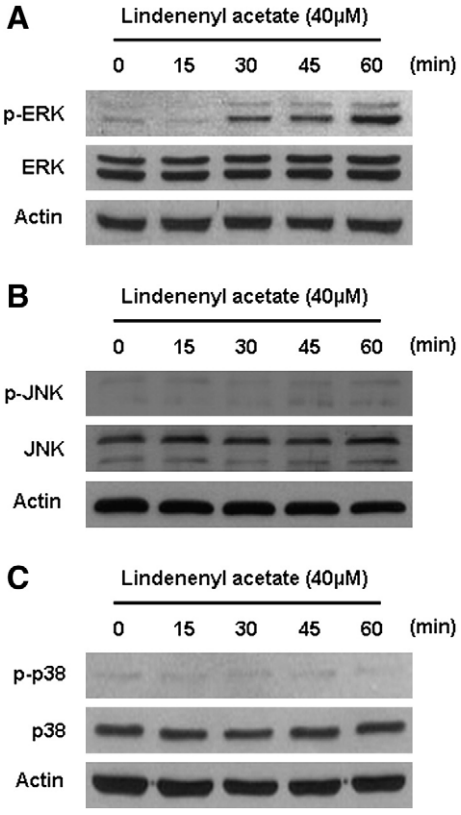

Fig. 6. Effects of lindenenyl acetate on ERK (A), JNK (B), and p38 MAPK (C) expression in HT22 cells. Cells were treated with 40 μM lindenenyl acetate for the indicated times. Cell extracts were analyzed by Western blot with antibodies specific for phosphorylated ERK1/2 (p-ERK), phosphorylated JNK (p-JNK), 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 three independent experiments are shown.

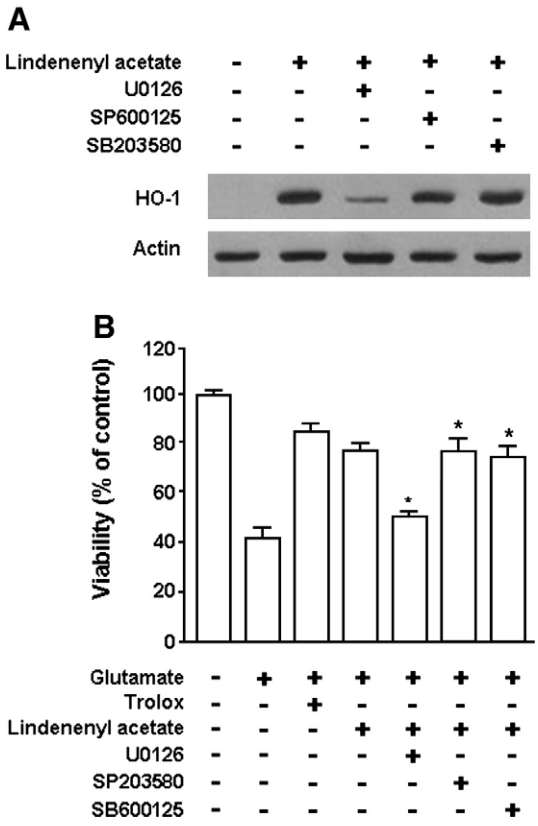

Fig. 7. Effects of lindenenyl acetate-induced ERK activation on HO-1 expression and glutamate-induced neurotoxicity. (A) Cells were incubated with 40 μM of lindenenyl acetate for 12 h in the presence or absence of U0126 (10 μM), SB203580 (20 μM), and SP600125 (25 μM). Western blotting was then performed with HO-1 antibody. (B) Cells untreated or treated with lindenenyl acetate (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 ± S.D. of three independent experiments. *$P<0.05$ vs. glutamate (5 mM). Trolox (50 μM) was used as positive control.

3.3. Effects of lindenenyl acetate on Nrf2 nuclear translocation and ARE activation

Several studies have shown that some genes encoding antioxidant enzymes, such as HO-1, have a specific sequence contained with their enhancer region, the antioxidant response element (ARE) (Nguyen et al., 2003; Alam et al., 1999). Furthermore, in the mechanism of HO-1 expression, nuclear translocation of activated Nrf2 is an important upstream contributor. Therefore, we investigated whether treatment of HT22 cells with lindenenyl acetate induced the translocation of Nrf2 to the nucleus. Using Western blot analysis, we tested the presence of Nrf2 proteins in nuclear compartments of HT22 cells. The cells were incubated with lindenenyl acetate for 0.5, 1 and 1.5 h at a concentration of 40 μM, and the nuclear fractions of lindenenyl acetate-treated cells showed a gradual increase in Nrf2 levels, while they declined concomitantly in the cytoplasm (Fig. 5A). In addition, HT22 cells transiently transfected with the ARE-luciferase plasmid were exposed to lindenenyl acetate, and changes in luciferase activity were used as a measure of ARE activation. The reporter assay showed that lindenenyl acetate dose-dependently increased ARE-driven luciferase activity

(Fig. 5B), and this ARE activation strongly correlated with the increase in heme oxygenase activity (Fig. 2C).

3.4. Involvement of the ERK pathway in lindenenyl acetate-induced HO-1 expression

MAPK is activated in response to oxidative stress and other various stressors. Several studies have reported that the activation of the MAPK pathways contributes to the induction of HO-1 (Kietzmann et al., 2003; Choi et al., 2004). Therefore, we examined the effect of lindenenyl acetate on activation of MAPKs in HT22 cells. Cells were exposed to lindenenyl acetate, total protein was harvested, and then Western blots were performed using anti-phospho-c-Jun NH2-terminal kinase (JNK), extracellular signal-regulated kinase 1/2 (ERK1/2) and p38 antibodies. At a concentration of 40 μM, which strongly induced the levels of HO-1, lindenenyl acetate activated the ERK pathway and increased ERK phosphorylation. As shown in Fig. 6A, phosphorylation of ERK was observed 30 min after lindenenyl acetate treatment, and was sustained for up to 60 min after lindenenyl acetate treatment. In contrast, phosphorylation of JNK and P38 kinases did not show at all the tested time periods (Fig. 6B and C). Furthermore, to investigate the role of MAPK in HO-1 expression, and to test the cytoprotective effects of lindenenyl acetate, we examined the effects of specific inhibitors of ERK1/2 (U0126), JNK (SP600125), and p38 (SB203580) on the levels of HO-1, by Western blot and MTT assay. The ERK MAPK pathway inhibitor significantly reduced lindenenyl acetate-induced HO-1 expression, whereas the JNK and p38 inhibitors did not (Fig. 7A). As expected, the ERK MAPK pathway inhibitor abolished lindenenyl acetate-induced cytoprotection, but inhibitors of JNK or p38 MAPK pathways did not (Fig. 7B). The ERK inhibitor was not cytotoxic under our experimental conditions (data not shown).

3.5. Involvement of the PI3K/Akt pathway in lindenenyl acetate-induced HO-1 expression

In the previous studies, phosphatidylinositol 3-kinase (PI3K) was involved in the expression HO-1 induced by various phytochemicals (Choi and Kim, 2008; Martin et al., 2004; Wu et al., 2006). Therefore, we tested whether lindenenyl acetate-induced expression of HO-1 occurs through the PI3K pathway. Fig. 8A showed that pretreatment with 10 μM LY294002 (a specific inhibitor of PI3K) significantly attenuated HO-1 expression induced by lindenenyl acetate in HT22 cells. Moreover, to correlate the activation of Akt with the induction of HO-1 by lindenenyl acetate, we investigated Akt phosphorylation in HT22 cells using an anti-phospho-Akt antibody. The phosphorylation of Akt was observed from 10 to 40 min and slowly declined thereafter (Fig. 8B). Pretreatment of the cells with LY294002 attenuated the phosphorylation of Akt and ERK in response to lindenenyl acetate (Fig. 8C and D). Furthermore, the PI3K pathway inhibitor (LY294002) abolished lindenenyl acetate-induced cytoprotection (Fig. 8B). Therefore, we suggested that lindenenyl acetate-induced expression of HO-1 was mediated through the PI3K/Akt pathway in HT22 cells.

4. Discussion

Neuronal cell death is an important phenomenon in mammalian neuronal development, and oxidative stress or the accumulation of reactive oxygen species is associated with many forms of apoptosis, as well as cell death that occurs in ischemia, trauma, and other neurodegenerative disease conditions (Coyle and Puttfarcken, 1993; Satoh et al., 1999; 2006; Satoh and Lipton, 2007). In addition, oxidative glutamate toxicity has been observed in neuronal cells including primary neuronal cells and tissue slices (Oka et al., 1993; Murphy

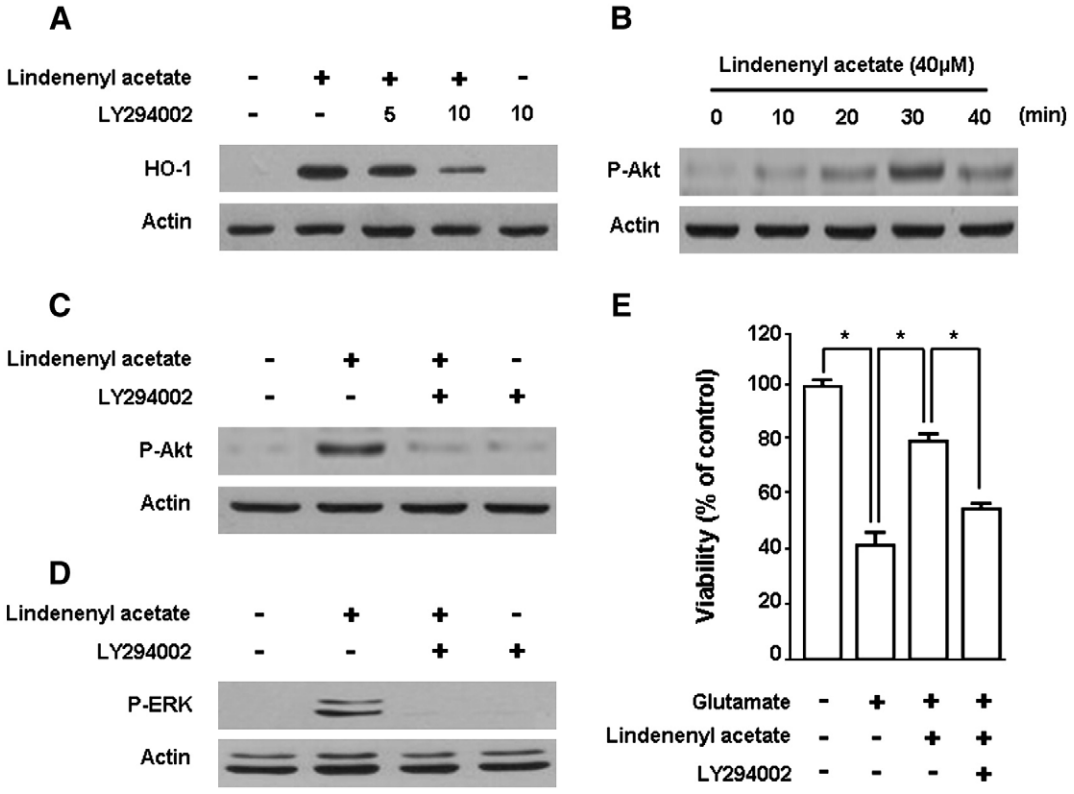

Fig. 8. Effects of lindenenyl acetate-induced HO-1 expression through the PI3K/AKT cascade. (A and C) Cells were pre-incubated with or without 10 μM of LY294002 for 1 h, and were incubated in the absence or presence of 40 μM of lindenenyl acetate for 12 h (A) or 30 min (C). (B) Cells treated with lindenenyl acetate (40 μM) for the indicated times. (D) Cells were pre-incubated with or without 10 μM of LY294002 for 30 min. Cell extracts were analyzed by Western blot with specific antibodies, and representative blots of three independent experiments are shown. (E) Cells untreated or treated with lindenenyl acetate (40 μM) in the presence or absence of LY294002 (10 μM) for 12 h were exposed to 5 mM glutamate for 8 h. Each bar represents the mean ± S.D. of three independent experiments, $^{*}P<0.05$.

et al., 1989; Vornov and Coyle, 1991). Therefore, glutamate-induced oxidative damage is a major contributor to pathological cell death within the nervous system (Coyle and Puttfarcken, 1993). In this respect, naturally occurring compounds that have intrinsic anti-oxidative effects against glutamate-induced oxidative stress, and which can trigger the intracellular cascade of protective pathways, may offer a promising strategy for therapeutic applications. In our previous studies, certain phytochemicals were reported to protect immortalized mouse hippocampal HT22 cells against glutamate-induced oxidative damage (Jeong et al., 2007, 2008). As a part of our continuing research to identify phytochemicals that can protect HT22 cells, we have discovered lindenenyl acetate, isolated from the roots of *L. strychnifolia*, as possessing protective effects against glutamate-induced oxidative damage in HT22 cells. The present investigation demonstrated that lindenenyl acetate increased the cellular resistance of HT22 cells to oxidative injury caused by glutamate, through Nrf2/ARE-dependent HO-1 expression, via activation of the ERK pathway.

Generally, the cytoprotective properties of antioxidants have been partially attributed to their abilities to induce cytoprotective enzymes. The expression of HO-1, a critical cytoprotective enzyme in response to oxidative injury, is considered to be an adaptive and protective response against oxidative insult in a wide variety of cells (Kietzmann et al., 2003; Satoh et al., 2003; Choi et al., 2002), including neuronal cells such as HT22 (Rössler et al., 2004; Suh et al., 2007; Jeong et al., 2008). We have provided evidence for the induction of HO-1 by lindenenyl acetate in HT22 cells, and showed that lindenenyl acetate-induced HO-1 protein expression and HO activity occurred in a concentration- and time-dependent manner (Fig. 2). Furthermore, pre-incubation of HT22 cells with lindenenyl acetate resulted in enhanced resistance to glutamate-induced oxidative damage; this effect was partly attributable to HO-1 expression as the SnPP, which inhibits HO enzyme activity, significantly reduced the cytoprotection afforded by lindenenyl acetate (Fig. 4). The induction of HO-1 expression was also required to suppress glutamate-induced reactive oxygen species generation (Fig. 4). These results strongly indicate that in our experimental setting, the observed cytoprotective effects of lindenenyl acetate were mediated through HO-1 expression.

In recent reports, it has been suggested that phytochemicals can activate Nrf2 by directly binding to Keap1 through covalent linkage, resulting in the induction of some cytoprotective proteins including HO-1 (Balogun et al., 2003; Haridas et al., 2004). The Nrf2 transcription factor plays a vital role in the ARE-mediated expression of phase 2 detoxifying and antioxidant enzymes, and in the activation of other inducible genes by various stimuli in response to oxidative stress (Itoh et al., 1997). The Nrf2 is required for the expression of some inducible proteins, such as GSH *S*-transferase, quinine reductase and HO-1 (Alam et al., 1999; Jaiswal, 2000). In the present study, we showed that lindenenyl acetate significantly induced the levels of Nrf2 and efficiently promoted its translocation into the nucleus (Fig. 5). In addition, we report that translocation of Nrf2 to the nucleus following lindenenyl acetate treatment was associated with an increase in its ARE transcriptional activity (Fig. 5). Therefore, this suggests that Nrf2 may play an essential role in lindenenyl acetate-induced HO-1 expression.

Furthermore, activation of the ERK pathways appeared to be involved in lindenenyl acetate-induced HO-1 expression (Fig. 6). Treatment of cells with specific protein kinase inhibitors demonstrated that the ERK pathway played crucial functions in the induction of HO-1. It has previously been observed that the MAPK pathways played a regulatory role in *HO-1* gene expression (Kietzmann et al., 2003; Elbirt et al., 1998). However, the exact roles of the MAPK pathways in the induction of HO-1 protein remain controversial.

Similar to the results of the present study, the activation of ERK was found to be required for the induction of HO-1 in several cells treated with diallyl sulfide (Gong et al., 2004), arsenite (Elbirt et al., 1998) and brazilin (Choi and Kim, 2008). This may be due to the diverse assortment and intensity of the signaling pathways activated by different inducers in different cell types. Lindenenyl acetate-induced HO-1 expression directly correlated with the ERK pathway because the inhibitor, U0126, blocked the expression completely. As expected, the ERK pathway inhibitor abolished lindenenyl acetate-induced cytoprotection, but inhibitors of the JNK or p38 MAPK pathways did not (Fig. 7). Our findings were similar to those shown in the study by Chen et al. (2005). These investigators showed that resveratrol induced HO-1 expression via activation of ERK and Nrf2–ARE signaling in PC12 cells. Furthermore, we also demonstrated the activations of the PI3K/Akt and ERK pathways were involved in lindenenyl acetate-induced HO-1 expression (Figs. 7 and 8). Specific protein kinase inhibitors attenuated the activations of the PI3K/Akt and ERK pathways and cytoprotective effects by lindenenyl acetate in HT22 cells. Choi and Kim (2008) reported that brazilin increased HO-1 expression through the activation of PI3K/Akt cascade and ERK pathways. Our results also indicate that PI3K/Akt cascade and ERK pathways, which are activated by lindenenyl acetate, participate in an early stage of HO-1expression in HT22 cells. However, further studies are extensively ongoing to define the exact role of the PI3K/Akt cascade and MAPK pathways in HO-1 expression.

In conclusion, the present results suggest that lindenenyl acetate effectively prevents glutamate-induced oxidative damage; HO-1 induction by lindenenyl acetate via ERK and Nrf2 pathways appear to play key roles in the protection of HT22 cells. Our results provide insight into the mechanisms of lindenenyl acetate-induced neuronal cytoprotection via the induction of HO-1, and suggest possible strategies for neuroprotection.

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