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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

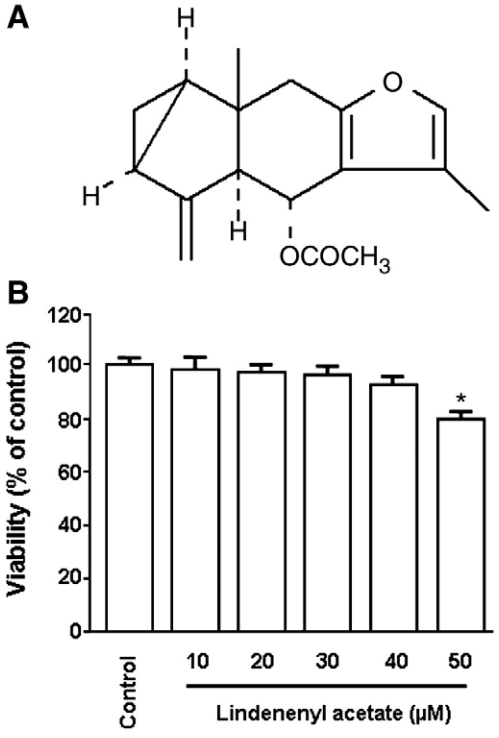

Fig. 1. The structure of lindenenyl acetate (A) and effects of lindenenyl acetate on cell viability; (B) HT22 cells were incubated for 12 h with various concentrations of lindenenyl acetate (10–50 μM). Cell viability was determined as described in the Materials and methods section. Each bar represents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