

Original Article

Isochaihulactone protects PC12 cell against H₂O₂ induced oxidative stress and exerts the potent anti-aging effects in *D*-galactose aging mouse model

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Aim: To investigate the effect of isochaihulactone (also known as K8), a lignan compound of *Bupleurum scorzonerifolium*, on H₂O₂-induced cytotoxicity in neuronally differentiated PC12 cells (nPC12).

Methods: Viability of neuronal PC12 cells was measured using MTT assay. Protein expression was determined by Western blot. Apoptotic cells was determined using TUNEL assay. *D*-galactose aging mice were used as a model system to study the anti-oxidant effects of isochaihulactone *in vivo*.

Results: Pretreatment with isochaihulactone (5–10 μmol/L) increased cell viability and decreased membrane damage, generation of reactive oxygen species and degradation of poly (ADP-ribose) polymerase in H₂O₂-treated nPC12 cells and also decreased the expression of cyclooxygenase-2, via downregulation of NF-κB, resulting in a decrease in lipid peroxidation. The results suggest that isochaihulactone is a potential antioxidant agent. In a murine aging model, in which chronic systemic exposure to *D*-galactose (*D*-gal) causes the acceleration of senescence, administration of isochaihulactone (10 mg·kg⁻¹·d⁻¹, sc) for 7 weeks concomitant with *D*-gal injection significantly increased superoxide dismutase and glutathione peroxidase activities and decreased the MDA level in plasma. Furthermore, H&E staining to quantify cell death within hippocampus showed that percentage of pyknotic nuclei in the *D*-gal-treated mice were much higher than in control.

Conclusion: The results suggest that isochaihulactone exerts potent anti-aging effects against *D*-gal in mice possibly via antioxidative mechanisms.

Keywords: lignan; isochaihulactone; neuroprotection; cyclooxygenase-2; anti-aging; PC12 cells; *D*-galactose; aging

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Introduction

Oxidative stress is believed to be a primary factor in neurodegenerative diseases as well as in the normal process of aging^[1–3]. Oxygen-derived free radicals exert detrimental effects including peroxidation of membrane lipids, enzyme inactivation, DNA fragmentation and activation of apopto-

sis^[4–6]. Superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) act by scavenging the superoxide anion and H₂O₂ to prevent reactive oxygen species (ROS)-induced damage^[7]. Exogenous H₂O₂ can increase oxidative stress and apoptotic cell death by causing mitochondrial dysfunction and activation of caspases. Therefore, H₂O₂ has been used extensively as an inducer of oxidative stress in *in vitro* models.

Reactive oxygen species themselves can increase and/or induce cellular cyclooxygenase-2 (COX-2) expression^[8–10]. In addition, apoptotic cell death induced by exposure to cyanide

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can be inhibited by selective COX-2 inhibition^[11, 12]. Oxidative stress-induced COX-2 expression can be prevented in numerous cell types, including neurons, by free radical scavengers. Thus, oxidant stressors are specific and important inducers of COX-2 gene expression.

The free radical theory of aging was conceived by Harman in 1956. Increasing evidence has convinced many researchers that oxidants play an important role in aging. Chronic administration of a low dose of *D*-galactose (*D*-gal) induces changes that resemble natural aging in animals^[13-20]. *D*-galactose is a physiological nutrient obtained from lactose in milk. The hydrolysis of lactose in the intestine results in monosaccharide glucose and galactose. In animals, galactose is normally metabolized by *D*-galactokinase and galactose-1-phosphate uridylyltransferase but over-supply of *D*-galactose results in abnormal metabolism (Kaplan and Pesce, 1996). *D*-galactose was converted into galactitol, which is not metabolized by above enzymes but accumulates in the cell, that leads to osmotic stress and ROS production^[21]. In addition, supplementation with antioxidants has been reported to be beneficial with respect to slowing the aging process^[22, 23].

Nan-Chai-Hu (Chai Hu of the South), the root of *Bupleurum scorzonerifolium*, is an important Chinese herb^[24]. Isochaihulactone (also known as K8) is a lignan compound that was identified in acetone extracts of Nan-Chai-Hu and shows anti-tumor activity against A549 cells *in vitro* and *in vivo*^[25, 26]. Lignan compounds (eg, sesamin, sesamol) have been reported to act as neuroprotective agents against oxidative injury and excitotoxicity^[27-30]. Lignans can also inhibit lipopolysaccharide-inducible COX-2 expression in macrophages^[31]. The aim of the present study was to investigate the effects of isochaihulactone on H₂O₂-induced injury in neuronal PC12 cells (nPC12) and in a murine *D*-gal-induced aging model.

Materials and methods

Fraction purification of isochaihulactone and structure determination

Bupleurum scorzonerifolium roots were supplied from Chung-Yuan Co, Taipei, and the plant was identified by Professor Lin of the National Defense Medicinal Center, where a voucher specimen was deposited (NDMCP No 900801). The acetone extract AE-BS was prepared as described previously^[25, 32]. The AE-BS was dissolved in 95% MeOH solution and then partitioned (1:1) with *n*-hexane to give the *n*-hexane-soluble fraction (BS-HE). The aqueous 95% MeOH layer was evaporated to remove residual MeOH, and then distilled water was added. This aqueous solution was further partitioned with CHCl₃ to get the CHCl₃-soluble fraction (BS-CE) and H₂O-soluble fraction (BS-WE). The BS-CE was subjected to chromatography over silica gel and eluted with CH₂Cl₂, CH₂Cl₂-MeOH (95:5), CH₂Cl₂-MeOH (9:1), CH₂Cl₂-MeOH (8:2) and MeOH, successively. The bioactive component, BS-CE-E02, was applied to silica gel and eluted with CH₂Cl₂-MeOH (99:1) to obtain isochaihulactone. The pure compound, isochaihulactone forms white needle crystals with physical properties of mp 137-138 °C; [α]_D²⁵ 29.08 (ca 0.5, CHCl₃); IR (KBr) ν_{max} cm⁻¹: 1745,

1635, 1581, 1335, 1153; UV (CHCl₃) λ_{max} nm (log ε): 247 (4.08), 298 (4.17), 327 (4.08); ¹H NMR (CDCl₃) δ: 3.29 (1H, m, H-3), 4.10 (1H, dd, J=9.0, 3.8 Hz, H-4a), 4.31 (1H, dd, J=9.0, 7.3 Hz, H-4b), 6.60 (1H, d, J=1.5 Hz, H-5), 2.78 (1H, dd, J=13.7, 9.0 Hz, H-6a), 2.92 (1H, dd, J=13.7, 6.7 Hz, H-6b), 7.24s (2H, s, H-20, 60), 6.67 (1H, d, J=1.4 Hz, H-200), 6.74 (1H, d, J=7.8 Hz, H-500), 6.61 (1H, dd, J=7.8, 1.4 Hz, H-600), 3.87 (9H, s, OMe), 5.93 (1H, d, J=1.3 Hz, OCH₂O), 5.94 (1H, d, J=1.3 Hz, OCH₂O); ¹³C NMR (CDCl₃) δ: 169.29s (C-1), 126.36s (C-2), 44.43d (C-3), 69.82t (C-4), 140.60d (C-5), 40.72t (C-6), 128.83s (C-10), 108.65d (C-20), 152.62s (C-30), 139.61s (C-40), 152.62s (C-50), 108.65d (C-60), 131.31s (C-100), 109.29d (C-200), 147.94s (C-300), 146.49s (C-400), 108.39d (C-500), 122.29d (C-600), 56.18q (20-OMe), 60.90q (30-OMe), 56.18q (40-OMe), 101.03t (OCH₂O); EIMS, 70 eV, *m/z* (rel int): 398 ([M]⁺, 18), 263 (100), 207 (16), 135 (35).

Chemicals and reagents

Isochaihulactone was dissolved in DMSO to a concentration of 100 mmol/L and stored in -20 °C as a stock solution. Dimethyl sulfoxide (DMSO), 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), 2',7'-dichlorofluorescein diacetate (H₂DCF-DA), Hoechst 33342, thiobarbituric acid (TBA), hydrogen peroxide (H₂O₂), trichloroacetic acid (TCA), malondialdehyde (MDA), propidium iodide (PI) and actin antibody were purchased from Sigma Chemical Co (St Louis, MO, USA). F-12 medium, horse serum, fetal bovine serum (FBS), penicillin, streptomycin, trypsin/EDTA and NuPAGE Bis-Tris Electrophoresis System (pre-cast polyacrylamide mini-gel) were purchased from Invitrogen (Carlsbad, CA, USA). CellBIND surface dishes and mouse 2.5S nerve growth factor (NGF) were purchased from Millipore (Bedford, MA). COX-2 antibody was purchased from Thermo scientific (Waltham, MA, USA). PARP antibodies and horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG secondary antibodies were purchased from Cell signaling (MA, USA). Polyvinylidene difluoride (PVDF) membranes, BSA protein assay kit and Western blot chemiluminescence reagent were purchased from Amersham Biosciences (Arlington Heights, IL). Superoxide dismutase activity assay kit was purchased from BioVision (Mountain View, CA). Glutathione peroxidase assay kit was purchased from Cayman Chemical (MI, USA). DNA Fragmentation Assay Kit was purchased from Clontech Laboratories (Mountain View, CA). Non-Radioactive Cytotoxicity Assay Kit was purchased from promega (Madison, WI, USA).

Cell culture and differentiation of neuronal PC12 cells

Undifferentiated rat pheochromocytoma cells (PC12 cells) were obtained from the Bioresources Collection and Research Center (BCRC, Hsin Chu, Taiwan) and maintained in F-12 medium supplemented with 2.5% fetal bovine serum and 12.5% horse serum in a CO₂ incubator at 37 °C. To induce neuronal differentiation, PC12 cells grown on CellBIND surface dishes were incubated in the presence of 50 ng/mL of mouse 2.5S nerve growth factor (NGF). Experiments were carried out 72 h after NGF incubation while the percentage of neurite-bearing cells was added up to 80%-90%.

Establishment of *D*-galactose aging animal model

Male adult C57BL/6 mice were purchased from National Sciences Council (Taipei, Taiwan) weighing 28–30 g at the beginning of the experiment were used. Animals were randomly divided into three groups (control, *D*-gal-administration, and *D*-gal-administration plus isochaihulactone 10 mg/kg treatment) and maintained at 20 °C, 12 h light/12 h dark cycle with free access to food and water. *D*-Gal (100 mg/kg) was injected subcutaneously (sc) daily into mice for 7 weeks. Isochaihulactone (10 mg/kg body weight) was injected subcutaneously (sc) 3 h prior to *D*-Gal injection. All control animals were given saline. The plasma of each group were collected for MDA content, antioxidative enzyme activity analysis.

Growth inhibition assay

Cell viability was assessed by measuring formazan produced by the reduction of MTT. Neuronal PC12 cells in 96-well plates were treated with H₂O₂ and incubated for 24 h at 37 °C. Isochaihulactone was added 3 h to the culture prior to H₂O₂ addition. The cells in each well were then incubated in culture medium with 500 mg/mL MTT for 2 h. Absorbance at 570 nm of the maximum was detected by a Spectramax Microplate ELISA Reader (Molecular Devices Corp, Sunnyvale, CA).

Cytotoxicity analysis

Lactate Dehydrogenase (LDH) Release Assay is used to measure cell membrane damage as a function of the amount of cytoplasmic LDH released into the medium. The LDH assay is based on the reduction of NAD⁺ by the action of LDH. The generated NADH is utilized for stoichiometric conversion of tetrazolium dye. LDH activity can be used as an indicator of cytotoxicity. Neuronal PC12 cells in 96-well plates were treated with H₂O₂ and incubated for 24 h at 37 °C. The 100 μmol/L α-tocopherol was used as a positive control (PC). Isochaihulactone or 100 μmol/L α-tocopherol was added 3 h to the culture prior to H₂O₂ addition, and LDH content was determined using the Non-Radioactive Cytotoxicity Assay (Promega). The test was performed according to the manufacturer's protocol. Briefly, at the end of the incubation, an aliquot of the medium (50 μL) was added to the kit reagent and incubated for 30 min, and then the reaction was stopped and the absorbance was measured at 490 nm using a microplate reader.

In situ TdT-mediated dUTP nick end labeling (TUNEL) assay

Apoptotic cells were confirmed with the DNA Fragmentation Assay Kit (clontech), in accordance with the manufacturer's instructions. Neuronal PC12 cells in 96-well plates were treated with H₂O₂ and incubated for 24 h at 37 °C. Isochaihulactone was added 3 h to the culture prior to H₂O₂ addition, then cells were fixed in 4% paraformaldehyde for 25 min at 4 °C, and then permeabilized with 0.2% Triton X-100 for 5 min at room temperature. Free 3' ends of fragmented DNA were enzymatically labeled with the TdT-mediated dUTP nick end labeling (TUNEL) reaction mixture for 60 min at 37 °C in a humidified chamber. Cell nuclei was monitored by Propidium

Iodine (PI) staining. Labeled DNA fragments were monitored by fluorescence microscopy (Zeiss).

Hoechst 33342 staining

After a 24 h treatment of the cells with H₂O₂ (200 μmol/L), Hoechst 33248 staining was performed. Isochaihulactone was added 3 h prior to H₂O₂ stimulation. Neuronal PC12 cells were stained with Hoechst 33248 dye to evaluate apoptosis. The cells were fixed with 4% paraformaldehyde at room temperature and stained with Hoechst 33342 working solution (5 μmol/L) for 30 min, then washed with PBS. Fluorescence was visualized using a fluorescent microscope (Zeiss) under 200× magnification.

Intracellular reactive oxygen species detection

The production of intracellular reactive oxygen species was estimated by using a fluorescent probe, 2',7'-dichlorofluorescein diacetate (DCFH-DA). DCFH-DA is transported across the cell membrane and hydrolyzed by intracellular esterases to form non-fluorescent 2',7'-dichlorofluorescein (DCFH), which is then rapidly converted to highly fluorescent 2',7'-dichlorofluorescein (DCF) in the presence of reactive oxygen species. The DCF fluorescence intensity is believed to be parallel to the amount of reactive oxygen species formed intracellularly. After 24 h treatment with 200 μmol/L H₂O₂, Cells were collected and CH₂ DCFDA was added (final concentration 10 μmol/L) for 60 min at 37 °C. Cells were washed by PBS for at least three times. The production of reactive oxygen species was measured immediately by Cell lab Quanta™ SC Flow cytometer (Beckman coulter).

Measurement of MDA content and antioxidant enzyme activities

The content of MDA was determined using the thiobarbituric acid method. Equal volumes of 0.67 % thiobarbituric acid reagent was added to the sample supernatant and boiled for 10 min at 100 °C, and cooled, the absorbance of each supernatant was measured at 532 nm. MDA content was calculated by MDA standard. Antioxidant enzyme activities were assayed with Superoxide dismutase activity assay kit (BioVision) and glutathione peroxidase assay kit (Cayman). The assay was in accordance with the manufacturer's instructions.

RNA extraction and RT-PCR assay

Total RNA from each sample was isolated by RNeasy (Qiagen, Valencia, CA, USA), according to the manufacturer's specifications. RNA quality was assessed using agarose gel electrophoresis. The concentration was calculated spectrophotometrically and 1 μg of total-RNA from each sample was used to generate cDNA using the Omniscript RT kit (Qiagen) according to manufacturer's protocol. One micrograms of cDNA was amplified in the presence of 1 mmol/L primers: *cox2*: (F) 5'-ACACTCTATCACTGGCATCC-3' and (R) 5'-GAAGGGACACCCTTTCACAT-3', *cox1*: (F) 5'-TTTGCACAACACTTCACCCACCAG-3' and (R) 5'-AAACACCTCCTG-GCCACAGCCAT-3', *p50*: (F) 5'-GTCTCAAACCAAACAGC-CTCAC-3' and (R) 5'-CAGTGTCTTCTCGACATGGAT-3',

rela: (F) 5'-GTCTCAAACCAAACAGCCTCAC-3' and (R) 5'-CAGTGTCTTCTCGACATGGAT-3', *sod1*: (F) 5'-AAGGC-CGTGTGCGTGTGCTGAA-3' and (R) 5'-CAGGTCTCCAACATG CCTCT-3', *sod2*: (F) 5'-CAGAGGCACAATGTCACCTCCTC-3' and (R) 5'-TTTATGGCCACAGTTTCACAGAA-3' and *gapdh*: (F) 5'-TGAAGGTCG GAGTCAACGGATTTGGT-3' and (R) 5'-CATGTGGGCCATGAGGTCCACCAC-3', with Taq DNA polymerase. The thermal cycling profile was composed of an initial denaturation step at 95 °C for 10 min, 30 cycles of 30 s of denaturation at 95 °C, 30 s of annealing at 58 °C (*cox1*, *cox2*, and *gapdh*) or 52 °C (*sod1*, *sod2*, *rela*, and *p50*), and 1 min of extension at 72 °C, with a final 5 min extension step at 72 °C. The intensity of bands was analyzed by AC Imaging System (LS Image Acquisition Software, UVP).

Western blot analysis

Neuronal PC12 cells in 96-well plates were treated with H₂O₂ and incubated for 24 h at 37 °C. Isochaihulactone or 100 μmol/L α-tocopherol (PC) was added 3 h to the culture prior to H₂O₂ addition. The cells were lysed on ice with 200 μL of lysis buffer (50 mmol/L Tris-HCl, pH 7.5, 0.5 mol/L NaCl, 5mmol/L MgCl₂, 0.5% nonidet P-40, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mg/mL pepstatin, and 50 mg/mL leupeptin) and centrifuged at 13000×g at 4 °C for 20 min. The protein concentrations in the supernatants were quantified using a BSA Protein Assay Kit. Electrophoresis was performed on a NuPAGE Bis-Tris Electrophoresis System using 50 mg of reduced protein extract per lane. Resolved proteins were then transferred to polyvinylidene difluoride (PVDF) membranes. Filters were blocked with 5% non-fat milk overnight and probed with appropriate dilution of primary antibodies for 1 h at room temperature. Membranes were washed with three times with 0.1% Tween 20 and incubated with HRP-conjugated secondary antibody for 1 h at room temperature. All proteins were detected using Western LightningTM Chemiluminescence Reagent Plus and quantified using a densitometers.

Statistical analysis

The data represent means±SD. Statistical differences were analyzed using the Student's *t*-test. For the pairwise comparisons multiple samples, statistical differences were analyzed using the *t*-test to compare the specific pairs of groups in one-way ANOVA (LSD procedure). Values of *P*<0.05 were considered significant.

Results

Isochaihulactone protected nPC12 cells against H₂O₂-induced cytotoxicity and apoptosis

The viability of nPC12 cells in response to exposure to 200 μmol/L H₂O₂ for 24 h was significantly (*P*<0.05) decreased, to 71% of that of control cells. Cells were also pretreated with isochaihulactone (Figure 1) or 100 μmol/L α-tocopherol (a potent antioxidant) 3 h before the addition of H₂O₂^[33]. Pretreatment with isochaihulactone (5 μmol/L or 10 μmol/L) significantly (*P*<0.05) inhibited this decrease (Figure 2A), whereas 40 μmol/L isochaihulactone did not exert any protec-

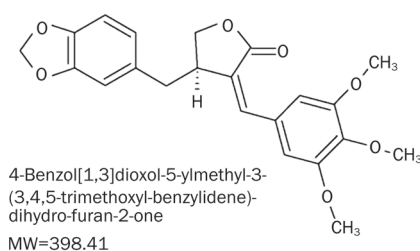


Figure 1. Chemical structure of isochaihulactone.

tive effect. To assess membrane damage, cells were treated with isochaihulactone (5 μmol/L or 10 μmol/L), and H₂O₂-induced cytotoxicity was determined by LDH assay. Treatment with H₂O₂ for 24 h showed an increase in LDH release compared to the control group, to 53.2%. Pretreatment with isochaihulactone (5 μmol/L or 10 μmol/L) significantly decreased LDH release, from 54.1% (vehicle-treated group) to 35.5% (5 μmol/L) and 27.6% (10 μmol/L). Pretreatment with 100 μmol/L α-tocopherol also significantly attenuated this increase in LDH release. There was no significant difference between the effect of isochaihulactone and that of α-tocopherol (Figure 2B).

We also assessed apoptosis in nPC12 cells by TUNEL assay, morphologic analysis of cell nuclei and poly (ADP-ribose) polymerase (PARP) degradation. Treatment of cells with H₂O₂ induced apoptosis, which was inhibited by pretreatment with isochaihulactone. In vehicle-treated control groups, cells were negative for TUNEL fluorescence. After exposure to 200 μmol/L H₂O₂ for 24 h, the percentage of TUNEL-positive cell increased. Pretreatment with isochaihulactone (10 μmol/L) for 3 h decreased the percentage of TUNEL-positive cells and significantly reduced apoptosis level back to control (Figure 2C). We next evaluated apoptosis via Hoechst 33342 staining to assess changes in nuclear morphology. As shown in Figure 2D, pretreatment with isochaihulactone (10 μmol/L) decreased the amount of chromatin condensation induced by H₂O₂. In addition, pretreatment with isochaihulactone for 3 h significantly (*P*<0.05) inhibited the H₂O₂-induced increase in caspase-3 and PARP activation (Figure 2E).

Isochaihulactone increased the antioxidant response of nPC12 cells

We assessed the level of intracellular ROS by DCFH-DA assay. Treatment of nPC12 cells with 200 μmol/L H₂O₂ for 24 h resulted in a 1.61-fold increase in intracellular ROS compared to vehicle-treated control cells. Coincubation with isochaihulactone (5 μmol/L or 10 μmol/L) significantly decreased ROS production compared to that in the vehicle-treated group (Figure 3A). Treatment with H₂O₂ markedly increased the level of the lipid peroxidation product MDA (Figure 3B) and decreased the antioxidant enzymatic activities of SOD and GPx (Figure 3C, 3D). Pretreatment with isochaihulactone (5 μmol/L or 10 μmol/L) resulted in a noticeable decrease in the MDA level and increased SOD and GPx activities compared

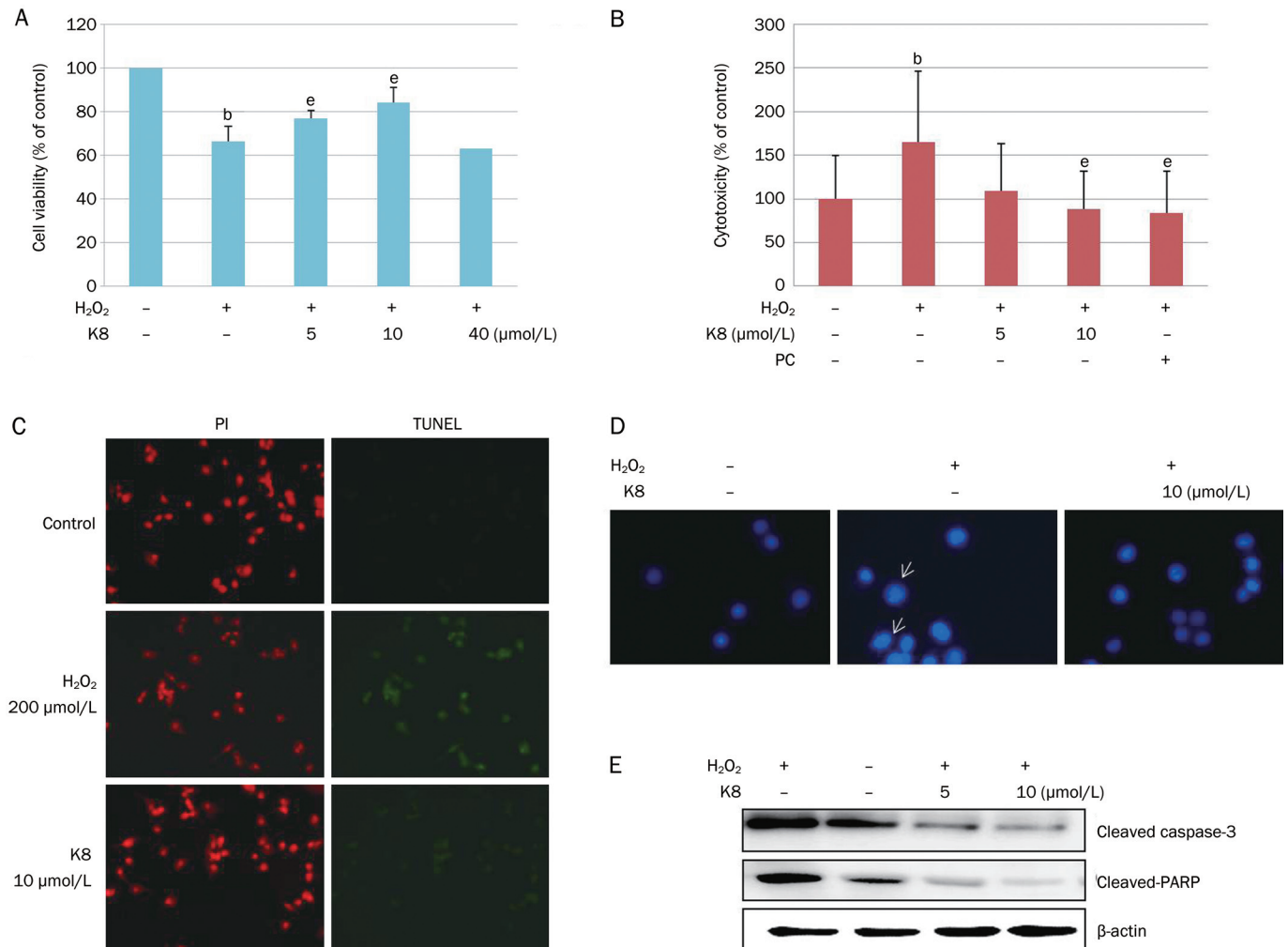


Figure 2. Attenuation of H₂O₂-induced injury cell by isochaihulactone in neuronally differentiated PC12 cells (nPC12). Isochaihulactone or 100 μmol/L α-tocopherol was added to the cultures 3 h before the addition of H₂O₂. Cells were incubated with 200 μmol/L H₂O₂ for 24 h for MTT, LDH or apoptosis assay. Pretreatment with isochaihulactone protected nPC12 cells against H₂O₂-induced injury by increasing cell viability (A) and decreasing H₂O₂-induced cytotoxicity. The 100 μmol/L α-tocopherol was used as a positive control (PC). (B) In addition, isochaihulactone (10 μmol/L) pretreatment decreased DNA fragmentation (C), chromatin condensation (D) Caspase-3 and PARP cleavage (E), apoptotic characteristics induced by H₂O₂. Data are presented as mean±standard deviation (SD) (n=3). ^bP<0.05 as compared to control group; ^eP<0.05 as compared to H₂O₂ treated group.

to those in the vehicle-treated group. In addition, SOD and GPx activities in nPC12 cells treated with isochaihulactone (5 μmol/L or 10 μmol/L) for 24 h showed no significant difference compared to control cells. Expression of SOD1 and SOD2 mRNA was downregulated in nPC12 cells in response to treatment with H₂O₂. Pretreatment with isochaihulactone inhibited this effect (Figure 3E), indicating that isochaihulactone not only elevated the activity of these antioxidant enzymes but also attenuated the decrease in SOD1 and SOD2 expression induced by H₂O₂.

Isochaihulactone inhibited COX-2 expression in H₂O₂-treated nPC12 cells

Reactive oxygen species can themselves increase cellular COX-2 expression. By Western blot analysis, pretreatment with isochaihulactone blocked H₂O₂-induced COX-2 mRNA

and protein expression in nPC12 cells but had no effect on COX-1 mRNA expression (Figure 4A, 4B). The transcription factor NF-kappa B is important in the regulation of COX-2 expression. Therefore, NF-kappa B mRNA expression was assessed after incubation of nPC12 cells with 200 μmol/L H₂O₂ for 3 h. mRNA expression of the NF-kappa B subunits P50 and RELA was downregulated by pretreatment with isochaihulactone (Figure 4C), indicating that isochaihulactone decreased the expression of COX-2 via downregulation of NF-kappa B.

Antioxidant effects of isochaihulactone in the D-galactose aging model

We measured the activities of T-SOD and GSH-Px and the MDA level in the plasma of mice. The MDA level in D-gal-treated mice was significantly increased compared to that

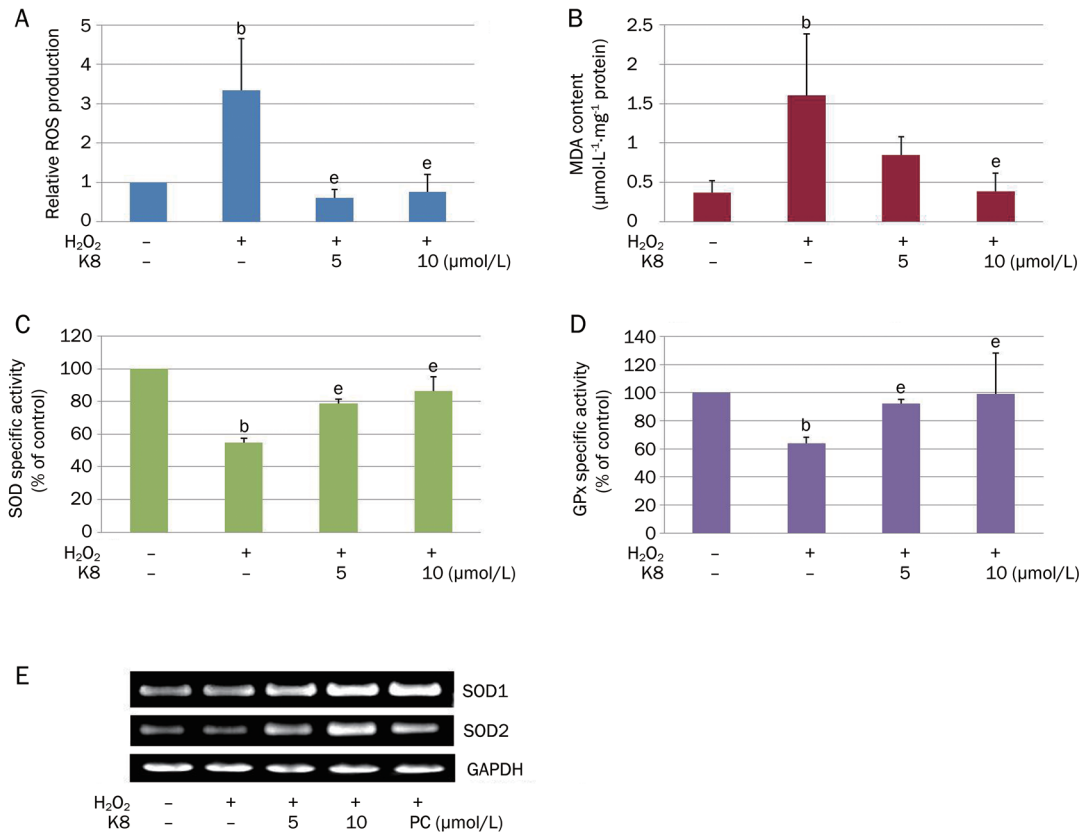


Figure 3. Effect of isochaihulactone on H₂O₂-induced intracellular accumulation of reactive oxygen species (ROS) and lipid peroxidation and downregulation of antioxidant enzyme (SOD and GPx) activity in neuronally differentiated PC12 cells (nPC12). Pretreatment with isochaihulactone attenuated the H₂O₂-induced accumulation of ROS (A) and lipid peroxidation (B). In addition, isochaihulactone (10 μmol/L) pretreatment maintained the activity of SOD (C) and GPx (D) as controls. Isochaihulactone also rescued mRNA transcription of SOD1 and SOD2, which was inhibited by H₂O₂ (E). Data are presented as mean ± standard deviation (SD) (n=3). ^bP<0.05 as compared to control group; ^eP<0.05 as compared to H₂O₂ treated group.

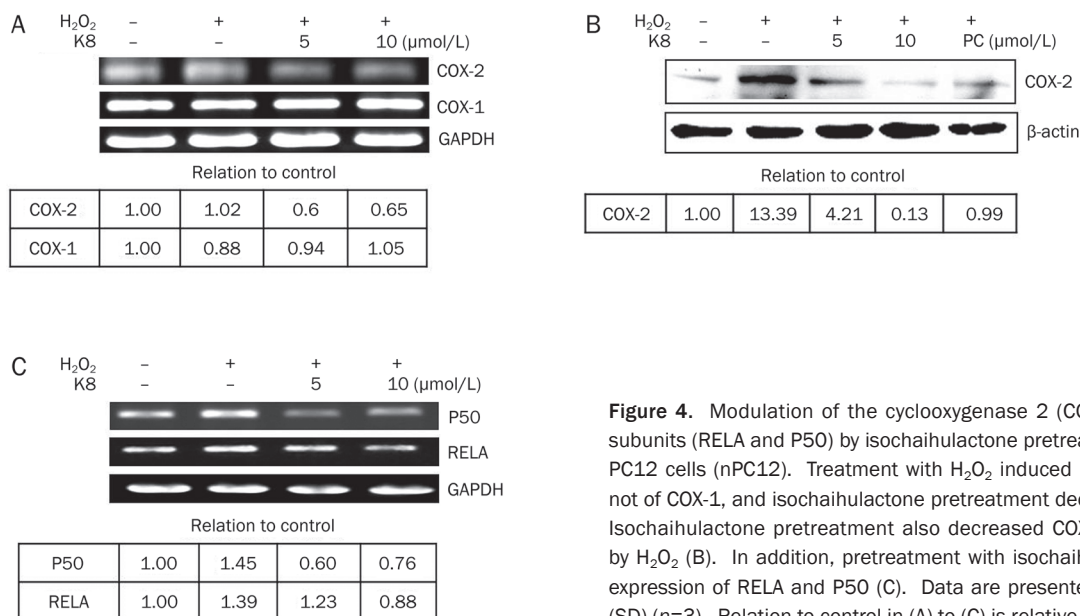


Figure 4. Modulation of the cyclooxygenase 2 (COX-2) isozyme and NF-kappa B subunits (RELA and P50) by isochaihulactone pretreatment in H₂O₂-treated neuronal PC12 cells (nPC12). Treatment with H₂O₂ induced mRNA expression of COX-2, but not of COX-1, and isochaihulactone pretreatment decreased this mRNA increase (A). Isochaihulactone pretreatment also decreased COX-2 protein expression induced by H₂O₂ (B). In addition, pretreatment with isochaihulactone decreased the mRNA expression of RELA and P50 (C). Data are presented as mean±standard deviation (SD) (n=3). Relation to control in (A) to (C) is relative to untreated control group.

in the control group (Figure 5A–5C). Administration of isochaihulactone ($10 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$) significantly inhibited this increase. The activities of T-SOD and GSH-Px in *D*-gal-treated mice were significantly decreased compared to those in the control group, and administration of isochaihulactone ($10 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$) significantly attenuated these decreases. Furthermore, we used H&E staining to quantify cell death within hippocampus: counting of pyknotic nuclei in H&E section. The result showed that percentage of pyknotic nuclei in the *D*-gal-treated mice were much higher than in control. Animals that received isochaihulactone showed a significantly decrease in the percentage of the damaged cells with respect to *D*-gal-receiving mice (Figure 5D).

Discussion

Results of the present study provide evidence that isochaihulactone can exert neuroprotective effects against H_2O_2 -induced oxidative stress in nPC12 cells. Pretreatment with isochaihulactone inhibited intracellular ROS formation. Although a small proportion of H_2O_2 may be scavenged by cellular antioxidant enzymes, it nonetheless directly induces the oxidation of various intracellular targets including the fluorescence probe DCFH-DA. When cells were exposed to exogenous H_2O_2 , DCF fluorescence increased significantly. The formation of hydroxyl radicals mediated by intracellular heavy metal ions may also contribute to the increased DCF fluorescence in response to H_2O_2 . Many reports indicate that lignans can access intracellular locations, owing to their benzylic structures, justifying their ability to attenuate oxidative stress induced by diverse stimuli^[34,35]. The chemical structure of isochaihulactone (sugar moiety attached to the 20 position of the

triterpene dammarane) may contribute to its direct antioxidant properties^[36]. However, antioxidant activity was also found in other cellular models, and the concentrations of isochaihulactone required for neuroprotection were far lower than those of H_2O_2 used in our present experiments, suggesting that it may not be a simple stoichiometric interaction.

Antioxidant activity of isochaihulactone was observed in the present study at concentrations of $5 \mu\text{mol/L}$ and $10 \mu\text{mol/L}$, whereas $40 \mu\text{mol/L}$ isochaihulactone showed no protective effects. In our previous study, we found isochaihulactone caused cytotoxicity in various cancer cell lines including lung, breast, ovary, colon, liver tumor cells ($\text{IC}_{50}=10\text{--}50 \mu\text{mol/L}$ after 48h), paclitaxel-resistant A549-T12 and P-gp-overexpression KB-TAX50 cells^[25]. In this study, we found that antioxidant activity of isochaihulactone was observed at concentrations of $5 \mu\text{mol/L}$ and $10 \mu\text{mol/L}$. These results revealed that isochaihulactone may activate different pathway through different concentration and cell types. Consistently, it has been reported that a major mammalian metabolite of plant-based lignans enterolactone act as antioxidants at relatively low concentrations with maximum protection at $100 \mu\text{mol/L}$ ^[37] and also used to induce anticancer activity of prostate cancer at higher than $100 \mu\text{mol/L}$ ^[38]. In addition, the PC12 cells used in the present study are clonal cells derived from rat pheochromocytoma. Treatment with nerve growth factor induces the differentiation of PC12 cells into a sympathetic neuron-like phenotype^[39]. This cell line has been used widely as a model in neurobiologic, neuropharmacologic and neurotoxicologic studies. The response of PC12 cells to isochaihulactone may not be exactly the same as that observed in other cells. Therefore, isochaihulactone exerts potent antiaging effects against

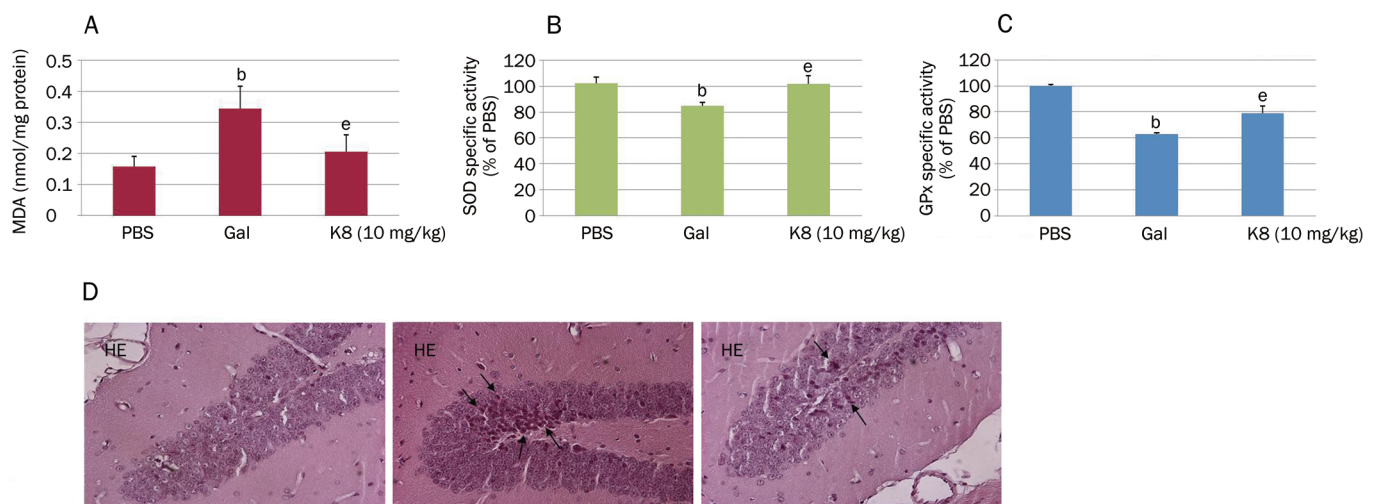


Figure 5. Effect of isochaihulactone on plasma MDA level and SOD and GPx activities in *D*-galactose-treated (aged) mice. The control group received subcutaneous (sc) injections of phosphate-buffered saline. The aged group received *D*-galactose (100 mg/kg , sc). The isochaihulactone group received *D*-galactose ($100 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$, sc) plus isochaihulactone ($10 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$, sc). Treatments were administered for 7 weeks. Isochaihulactone treatment attenuated the aging characteristics of increased MDA level and downregulated SOD and GPx activities. In addition, neuronal damage analysis. H&E staining shows that pyknotic nuclei in galactose-treated group (middle) were significantly increased compared with vehicle-treated group (left) and decreased in galactose+isochaihulactone treated group (right) compared with galactose alone group in the CA1 subfield of hippocampus after 7 weeks of administration (D). Data are presented as mean \pm standard deviation (SD) ($n=3$ mice). ^a $P<0.05$ as compared to control group; ^e $P<0.05$ as compared to H_2O_2 treated group.

D-gal in mice via antioxidative mechanisms at low dosage but a strong anti-proliferative effect at high dosage.

The cyclooxygenase (COX) enzymes catalyze a key step in the conversion of arachidonate to PGH₂, the immediate substrate for a series of cell specific prostaglandin and thromboxane synthases. There are two COX isoforms, which differ mainly in their pattern of expression. COX-1 is expressed in most tissues, whereas COX-2 usually is absent, but is induced by numerous physiologic stimuli. Results of the present study showed that isochaihulactone inhibited the expression of COX-2 and decreased lipid peroxidation. The dual intrinsic enzyme activities of COX-2 catalyze two sequential reactions in the metabolism of arachidonic acid (AA). The COX-2 enzyme possesses cyclooxygenase activity that metabolizes AA to hydroperoxide (PGG₂; 9,11-endo-peroxy-15-hydroperoxyprostaglandin) utilizing two oxygen molecules (2O₂), and it also possesses a heme-containing active site that provides peroxidase activity, which requires two electrons (2e⁻) to become active. The peroxidase reaction converts PGG₂ to PGH₂ by removing oxygen(s), [O_x], which may be a source of oxygen radicals. Therefore, as more AA is metabolized to PG by COX-2, more electron donors are depleted, and more oxygen radicals are generated. The COX-2-dependent production of ROS is likely to be involved in the enhanced lipid peroxidation in H₂O₂-treated cells. The mechanism for the induction of COX-2 in H₂O₂-induced apoptosis of nPC12 cells is unknown. The COX-2 inhibitor U0126 blocks hypoxia-induced MAPK/ERK1/2 activity in PC12 cells after 1 h of hypoxia and significantly protects against hypoxic death^[40], suggesting that COX-2 activation is involved in hypoxia in PC12 cells. Results of the present study showed that H₂O₂ increased the expression of COX-2 and the transcription factor p65 in nPC12 cells and that pretreatment with isochaihulactone inhibited this effect and decreased the level of LDH release in response to H₂O₂ treatment. This result indicates that isochaihulactone may also regulate MAPK signaling to protect nPC12 cells against H₂O₂-induced injury.

Many studies have shown that lignans possess potent antioxidant properties *in vitro* and *in vivo*. There have been no previous reports on the protective effect of isochaihulactone against *D*-gal-induced aging in mice. To protect cells against oxidative damage induced by ROS, the antioxidant system in the body is activated, and endogenous antioxidant enzymes, such as SOD and GPx, scavenge ROS or prevent their formation. The production of ROS can also be evaluated indirectly by analyzing the level of MDA, a product of free radical-induced lipid peroxidation. Analysis of the number of pyknotic nuclei cells in the hippocampus showed that isochaihulactone had an important protective effect against *D*-gal-induced cell death. Overall, our present findings suggest that isochaihulactone can protect mice against oxidative stress injury induced by *D*-gal and improves impairments in aging mice.

In conclusion, isochaihulactone decreased oxidative stress-induced ROS production and lipid peroxidation and also maintained endogenous antioxidant enzymatic activities, sta-

bilized mitochondrial function, and subsequently attenuated nPC12 cell injury. Although more detailed mechanistic studies are necessary to clarify the mechanism of neuroprotection by isochaihulactone, these results should encourage further studies to explore the potential neuroprotective effects of isochaihulactone in neurologic diseases.

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Author contribution

Yi-lin Sophia CHEN designed research; Sung-liang YU, Shih-bin LIN, Yung-luen YU, Min-hui CHIEN, Kuo-jung SU, Ching-ju LIN performed research; Tzong-der WAY, Giou-teng YIANG contributed new analytical tools and reagents; Chai-ching LIN, De-chuan CHAN, Horng-jyh HARN analyzed data; Sung-liang YU wrote the paper.

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