Available online at www.sciencedirect.com

SciVerse ScienceDirect

Journal of Nutritional Biochemistry 23 (2012) 1314–1323

Journal of Nutritional Biochemistry

Licochalcone E activates Nrf2/antioxidant response element signaling pathway in both neuronal and microglial cells: therapeutic relevance to neurodegenerative disease☆

Sa Suk Kim[a,1], Juhee Lim[a,1], Yeojin Bang[a], Jiyeong Gal[a], Sang-Uk Lee[a], Young-Chang Cho[a], Goo Yoon[b], Bok Yun Kang[a], Seung Hoon Cheon[a], Hyun Jin Choi[a,*]

[a]College of Pharmacy and Research Institute of Drug Development, Chonnam National University, Gwangju 500-757, South Korea
[b]College of Pharmacy, Mokpo National University, Jeonnam 534-729, South Korea

Received 17 February 2011; received in revised form 8 June 2011; accepted 27 July 2011

Abstract

Oxidative stress and neuroinflammation are hallmarks of neurodegenerative diseases, which do not play independently but work synergistically through complex interactions exacerbating neurodegeneration. Therefore, the mechanism that is directly implicated in controlling oxidative stress and inflammatory response could be an attractive strategy to prevent the onset and/or delay the progression of neurodegenerative diseases. The transcription factor nuclear factor-E2-related factor-2 (Nrf2) is the guardian of redox homeostasis by regulating a battery of antioxidant and phase II detoxification genes, which are relevant to defense mechanism against oxidative stress and inflammatory responses. In this study, we show that a recently identified *Glycyrrhiza-inflata*-derived chalcone, licochalcone E (Lico-E), attenuates lipopolysaccharide-induced inflammatory responses in microglial BV2 cells and protects dopaminergic SH-SY5Y cells from 6-hydroxydopamine cytotoxicity. Lico-E activates Nrf2-antioxidant response element (ARE) system and up-regulates downstream NAD(P)H:quinone oxidoreductase 1 (NQO1) and heme oxygenase-1 (HO-1). Anti-inflammatory and cytoprotective effects of Lico-E are attenuated in siRNA-mediated Nrf2-silencing cells as well as in the presence with specific inhibitor of HO-1 or NQO1, respectively. Lico-E also has neuroprotective effect against 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced nigrostriatal dopaminergic neurodegeneration in mice, with up-regulation of HO-1 and NQO1 in the substantia nigra of the brain. This study demonstrates that Lico-E is a potential activator of the Nrf2/ARE-dependent pathway and is therapeutically relevant not only to oxidative-stress-related neurodegeneration but also inflammatory responses of microglial cells both *in vitro* and *in vivo*.
© 2012 Elsevier Inc. All rights reserved.

Keywords: HO-1; Licochalcone E; Neuroprotection; NQO1; Nrf2; Parkinson's disease

1. Introduction

Parkinson's disease (PD) is a progressive neurodegenerative disease characterized by a selective loss of dopamine (DA)-producing neurons in the substantia nigra pars compacta (SNpc) and a reduction in their terminals within the striatum. Dopaminergic (DArgic) neurodegeneration in this nigrostriatal pathway causes typical motor deficits, such as resting tremor, rigidity, bradykinesia and postural instability. While the etiological link to DArgic neuronal loss remains unknown, evidences suggest crucial roles for oxidative stress, mitochondrial defects, protein aggregation and misfolding, and inflammation in the pathogenesis of PD [1,2].

Oxidative stress has been largely associated with the development of PD due to the highly oxidative conditions in DArgic neurons. Oxidative stress activates death signaling, including mitochondria-dependent apoptosis, and finally triggers cellular demise [3]. Among various candidates that can generate oxidative stress in DArgic neurons, DA itself is a primary source of oxidative stress that causes cytotoxicity to DArgic neurons [4]. DA-induced oxidative stress can be induced by auto-oxidation forming reactive quinone species [5–7]. Quinones are unstable and highly reactive and can readily attack cellular nucleophiles and cause covalent modification of essential macromolecules.

In addition, neuroinflammatory processes play a significant role in the generation of oxidative stress and the pathogenesis of PD. Neuropathological evidence indicates that neuroinflammatory response is detectable and accompanied by neuronal loss in PD: for example, inflammatory mediators are present in the cerebrospinal fluid [8] and the brains of PD patients [9], and signs of microglial activation can be observed in the area of SNpc in Parkinson's brain [10]. Many toxic factors, including superoxide anions, nitric oxide (NO), arachidonic acid and its metabolites, chemokines, and

☆ This research was supported by Technology Development Program for Agriculture and Forestry, Ministry for Food, Agriculture, Forestry and Fisheries, Republic of Korea (106073-1; to H.J. Choi).
* Corresponding author. Tel.: +82 62 530 2938; fax: +82 62 530 2911.
E-mail address: hjchoi3@chonnam.ac.kr (H.J. Choi).
[1] S.S. Kim and J. Lim contributed equally to this work.

0955-2863/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
doi:10.1016/j.jnutbio.2011.07.012

proinflammatory cytokines, can be generated by microglial activation. Activated microglia causes increased generation of reactive oxygen species (ROS), and oxidative stress also has the potential to induce an inflammatory response. Thus, these two mechanisms — oxidative stress and inflammation — are not independent but rather work synergistically through complex interactions exacerbating neurodegeneration. Therefore, the mechanism directly implicated in controlling oxidative stress and inflammatory response could be an attractive target in preventing the onset and/or delaying the progression of PD.

The transcription factor nuclear factor-E2-related factor-2 (Nrf2) is a central protein that regulates the transcription of phase II enzymes and other enzymes that are important in the antioxidative response. Under normal conditions, Nrf2 is sequestered in the cytoplasm by binding to Kelch-like ECH-associated protein 1 (Keap1). Upon stimulation, Nrf2 dissociates from Keap1 and is translocated into the nucleus where it binds to antioxidant response element (ARE) and activates the expression of ARE-dependent genes. ARE is a *cis*-acting regulatory element in promoter regions of genes encoding antioxidant proteins and plays a crucial role in the transcriptional regulation of downstream genes important in the cellular response to oxidative stress, such as NAD(P)H:quinone oxidoreductase 1 (NQO1) and heme oxygenase-1 (HO-1). NQO1 catalyzes the two-electron reduction of quinone to the redox-stable hydroquinone and protects cells against oxidative damage [11,12]. HO-1 is responsible for the conversion of heme to biliverdin and carbon monoxide and functions as an antioxidant enzyme [13,14] and an anti-inflammatory stress protein [15].

The roots of *Glycyrrhiza inflata* B. (Leguminosae) are known to be a source for characteristic phenolic compounds that have various pharmacological activities, including anti-inflammatory [16], antioxidative [17] and anticarcinogenic activities [18]. Six retrochalcones or reversely constructed chalcones have been isolated: licochalcone A–E and echinatin [18,19]. Licochalcone E (Lico-E) is a very recently characterized retrochalcone that exhibits potent cytotoxicity to human tumor cell lines and endothelial cells [20], as well as anti-inflammatory potential to reduce skin inflammation [21].

Because chalcones are powerful inducers of phase II detoxifying enzymes and cytoprotective HO-1 [22,23], in the present study, we asked whether (a) Lico-E possesses anti-inflammatory activity in lipopolysaccharide (LPS)-induced microglial BV2 cells, and antioxidant and cytoprotective activities from 6-hydroxydopamine (6-OHDA)-induced DArgic SH-SY5Y cell death; (b) Lico-E activates Nrf2 signaling and induces NQO1 and HO-1, which mediate the cytoprotective and anti-inflammatory activities of Lico-E; and (3) Lico-E is cytoprotective against DArgic neurodegeneration in the *in vivo* experimental models of PD.

2. Methods and materials

2.1. Materials

Lico-E was obtained from the roots of *G. inflata* B. [20]. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin/streptomycin and trypsin/EDTA were from Hyclone (Logan, UT, USA). LPS (L4516), 6-OHDA, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), α-actin antibody and TRI reagent were purchased from Sigma-Aldrich (St. Louis, MO, USA). Antibodies against inducible nitric oxide synthase (iNOS) (sc-649), cyclooxygenase-2 (COX-2) (sc-1745), β-actin (sc-8432), anti-Nrf2 (sc-722), NQO1 (sc-32793 and sc-16464), HO-1 (sc-10789) and tyrosine hydroxylase (TH; sc-25269) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and GAPDH (2118) was obtained from Cell Signaling Technology (Beverly, MA, USA). Enhanced Chemiluminescence (ECL) kit was from Amersham Biosciences (Piscataway, NJ, USA). All other chemicals were reagent grade and were purchased from Sigma-Aldrich or Merck (Rahway, NJ, USA).

2.2. Cell culture

BV2 mouse microglial cells and HEK293T cells were maintained in DMEM containing 10% FBS, and DA-producing SH-SY5Y cells were grown in DMEM/F12 containing 10% FBS. All cells were maintained in the presence of 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in 5% CO_2 and 95% air in a humidified atmosphere. For experiments, the cells were plated on polystyrene culture dishes at a density of 1.2×10^5 cells/well in 24-well culture plates or 3.6×10^6 cells/100-mm plate. After 24-h incubation, cells were fed with fresh medium for treatment.

2.3. Determination of NO levels

The level of NO synthesis was assessed by measuring nitrite accumulation in the culture media using Griess reagent (1% sulfanilamide, 0.1% *N*-1-naphthylenediamine dihydrochloride and 2.5% phosphoric acid). Briefly, 200-μl aliquots of cell culture medium were collected and mixed with an equal volume of Griess reagent. After incubation for 10 min, the optical density was measured at 540 nm, and nitrite levels were calculated from a standard curve generated with sodium nitrite.

2.4. Lactate dehydrogenase (LDH) assay

Degrees of cell death were assessed based on the activity of LDH released into the culture medium as previously described [24]. Aliquots (50 μl) of cell culture medium were incubated at room temperature in the presence of 0.26 mM NADH, 2.87 mM sodium pyruvate and 100 mM potassium phosphate buffer (pH 7.4) in a total volume of 200 μl. The rate of NAD^+ formation was monitored for 5 min at 2-s intervals at 340 nm using a microplate spectrophotometer (VERSAMAX 340 pc; Molecular Devices, Menlo Park, CA, USA).

2.5. Immunoblot analysis

For immunoblot analysis, cells were washed with PBS, harvested and lysed with RIPA buffer [50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1% Triton-X 100, 0.1% sodium dodecyl sulfate (SDS), 2 mM EDTA, pH 8.0] containing protease inhibitors. Equal amounts of protein were separated on 10% SDS-polyacrylamide gels and transblotted onto polyvinylidene difluoride-nitrocellulose filters. Membranes were incubated with anti-iNOS, COX-2, NQO1, HO-1, Nrf2 and TH and then incubated with horseradish-peroxide-conjugated secondary antibody, and specific bands were visualized using ECL detection kit. The blots were reprobed with anti-α-actin, -β-actin or -GAPDH antibodies to serve as a control for gel loading.

2.6. Nrf2 knockdown by siRNA transfection

For Nrf2 knockdown, BV2 or SH-SY5Y cells were transfected with Nrf2 siRNA using siRNA transfection reagent (Santa Cruz, CA, USA) according to the manufacturer's protocol. The sequences targeting Nrf2 correspond to the coding region nucleotides as follows: mouse Nrf2 (sense, 5′ GAA UUA CAG UGU CUU AAU A 3′; antisense, 5′ UAU UAA GAC ACU GUA AUU C 3′) and human Nrf2 (sense, 5′ CAA ACA GAA UGG UCC UAA A 3′; antisense, 5′ UUU AGG ACC AUU CUG UUU G 3′). For negative control, BV2 or SH-SY5Y cells were transfected with same concentration of control siRNA (Santa Cruz, CA, USA).

2.7. Reverse transcription polymerase chain reaction (RT-PCR) analysis

Total RNA was isolated using TRI reagent according to the manufacturer's protocol. To perform each RT-PCR, the RNA sample was reverse transcribed and amplified with gene-specific primers using the Maxime RT PreMix (oligo dT primer) and Maxime PCR PreMix (*i-StarTag*) Kit (iNtRON, Seongnamn, South Korea). The sequences of the synthetic oligonucleotides were as follows: mouse tumor necrosis factor-α (TNF-α) (sense, 5′ CAG ACC CTC ACA CTC AGA TCA TCT T 3′; antisense, 5′ CAG AGC AAT GAC TCC AAA GTA GAC CT), mouse interleukin-1β (IL-1β) (sense, 5′ ATG GCA ACT GTT CCT GAA CTC AAC T 3′; antisense, 5′ AGG ACA GGT ATA GAT TCT TTC CTT T 3′), mouse HO-1 (sense, 5′ CTA TGT AAA GCG TCT CCA 3′; antisense 5′ GTC TTT GTG TTC TCT GTC3′), human NQO1 (sense, 5′ AGG CTG GTT TGA GCG AGT 3′; antisense, 5′ ATT GAA TTC GGG CGT CTG CTG 3′), mouse β-actin (sense, 5′ CAT GTT TGA GAC CTT CAA CAC CCC 3′; antisense, 5′ GCC ATC TCC TGC TCG AAG TCT AG 3′) and human β-actin (sense, 5′ TCA CCC ACA CTG TGC CCA TCT ACG A 3′; antisense, 5′ GGA AGG CTG GAA GAG TGC CTC AGG G 3′). PCR was performed as described in Table 1. After amplification, 10 μl of the RT-PCR products was separated in 1.5% (w/v) agarose gels and stained with ethidium bromide.

2.8. Transient transfection and reporter gene assay

A dual-luciferase reporter assay system (Promega, WI, USA) was used to determine ARE-driven promoter activity. Cells were plated at a density of 1.2×10^5 cells/well in 24-well culture plates and transiently transfected with 0.5 μg of pGL-797 plasmid containing the ARE sequence [25] and 0.5 μg of pRL-TK plasmid using Hilymax according to the manufacturer's protocol (Dojindo Laboratories, Kumamoto, Japan) on the following day. After treatment with Lico-E, the firefly and renilla luciferase activities in the cell lysates were determined using a luminometer (Microlumat Plus LB 96V, BERTHOLD).

Table 1
RT-PCR conditions

	HO-1 (m)	Actin (m)	NQO1 (h)	TNF-α (m)	IL-1β (m)	Actin (h)
Initial denaturation	94°C/2 min					
Denaturation	94°C/30 s	94°C/20 s	94°C/20 s	94°C/30 s	94°C/30 s	94°C/30 s
Annealing	55°C/30 s	65°C/20 s	62°C/20 s	55°C/30 s	55°C/30 s	55°C/30 s
Extension	72°C/40 s	72°C/30 s	72°C/30 s	72°C/40 s	72°C/40 s	72°C/40 s
Final extension	72°C/5 min					
Cycle	30	30	38	30	30	30

m, mouse; h, human.

2.9. Immunocytochemistry

Cells were washed with PBS and fixed in cold 4% paraformaldehyde and 4% sucrose in PBS, pH 7.4, for 15 min. Cells were permeabilized and incubated for 1 h in blocking solution (0.3% Triton X-100, 1% bovine serum albumin, 1% normal goat serum in PBS) at room temperature. Cells were incubated with anti-Nrf2 in PBS containing 2% bovine serum albumin and 0.2% Triton X-100 for 1 h. Cells were then incubated with Alexa Fluor 488 rabbit anti-goat IgG (Molecular Probes, Eugene, OR, USA) at room temperature for 1 h. After incubation, nuclei were stained using Hoechst33258 for 5 min. Cell images were obtained by confocal microscopy.

2.10. Electrophoretic mobility shift assay (EMSA)

Oligonucleotides containing a nuclear factor (NF)-κB (5′CCG GTT AAC AGA GGG GGC TTT CG AG 3′) binding site were used as a probe. Specific binding was confirmed by a competition experiment with a 50-fold excess of unlabeled, identical oligonucleotides. The nuclear extracts (5 μg) were incubated with 1 μg of poly (dI-dC) and the [α-^{32}P]dCTP-labeled DNA probe in binding buffer (100 mM NaCl, 30 mM HEPES, 1.5 mM $MgCl_2$, 0.3 mM EDTA, 10% glycerol, 1 mM DTT, 1 mM PMSF and 1 μg/ml each of aprotinin and leupeptin) for 20 min on ice. The protein/DNA complex was separated from the free probe in a 4.8% polyacrylamide gel in 0.5×TBE buffer (44.5 mM Tris, 44.5 mM boric acid and 1 mM EDTA). After electrophoresis, the gel was dried and autoradiographed.

2.11. MPTP-induced Parkinson animal model

Male C57BL/6 mice (10–12 weeks, 20–25 g) were maintained in a temperature- and humidity-controlled room with a 12-h light–dark cycle with food and water available *ad libitum*. Animals were assigned to four groups with six to seven animals per group: control group, MPTP-treated control group, MPTP and Lico-E-treated group, and Lico-E-only-treated group. Lico-E was administered orally (10 mg/kg body weight, dissolved in 10% ethanol) five times at 24-h interval [26]. Two hours after the fifth administration of Lico-E, MPTP was administered (20 mg/kg body weight, 2-h interval, four times). Control animals received saline alone. Animals were killed by decapitation 7 days after the first injection of MPTP, and the whole brain was immediately frozen in liquid nitrogen and stored at −80°C for later analyses. To excise striatal and nigral tissues, 2-mm slices from bregma −2 mm to 0 mm and from bregma +2 mm to +4 mm using a brain atlas [6], respectively, were cut out in a brain matrix and excised using a micropunch with an inner diameter of 2 mm.

2.12. Data analyses

Comparisons were made using analysis of variance and unpaired Student's *t* test. $P<.05$ was considered statistically significant for all analyses.

3. Results

3.1. Lico-E attenuates LPS-induced inflammatory response in microglial BV2 cells

Microglia are resident macrophages in the central nervous system and are primarily responsible for the inflammatory response in the pathogenesis of various neurodegenerative diseases including PD. In the present study, we used the BV2 cell line, which is a well-known murine microglial cell line showing similar phenotypic and functional properties with reactive microglial cells [27].

Because a number of chalcone derivatives exhibit anti-inflammatory activity [28], we first asked whether Lico-E (Fig. 1) is able to reduce inflammatory responses in LPS-activated microglial cells. Activation of microglial cells leads to the induction of inflammatory enzymes such as iNOS and COX-2 and inflammatory cytokines such as IL-1β and TNF-α, and activation of transcription factor NF-κB. Fig. 2A shows that LPS increased generation of nitrite, a stable metabolite of NO, in the culture medium of BV2 cells (3.41±0.03 and 52.26±2.41 μM in the culture medium of control and LPS-treated cells at 24 h, respectively). Lico-E treatment significantly inhibited the accumulation of nitrite in the medium (3.53±0.23 μM in the culture medium of 5 μM Lico-E and LPS-treated cells at 24 h). The concentration of Lico-E leading to 50% inhibition (IC_{50}) of NO production was 2.25 μM at 24 h. Dexamethasone, a glucocorticoid that has anti-inflammatory activity [29,30], was used as a positive control. Lico-E did not induce cytotoxicity to BV2 cells at concentrations ranging from 1 to 5 μM in the presence or absence of LPS (Fig. 2B). The expressions of iNOS and COX-2 protein were dramatically up-regulated in LPS-treated BV2 cells (Fig. 2C), and this increase in the expression was also significantly attenuated by treatment with Lico-E (Fig. 2D). In addition, the mRNA levels of the inflammatory cytokines IL-1β and TNF-α were measured by RT-PCR analysis. The mRNA expression of IL-1β and TNF-α was barely detectable in untreated control cells and was increased in LPS-treated cells. As shown in Fig. 2E, 5 μM Lico-E completely abolished LPS-induced up-regulations of both IL-1β and TNF-α. NF-κB is a critical regulator of iNOS and COX-2 induction. Therefore, we examined the effect of Lico-E on NF-κB activation by LPS. EMSA data (Fig. 2F) showed that Lico-E inhibited NF-κB–DNA binding. All these data suggest that Lico-E attenuates LPS-induced inflammatory responses in microglial BV2 cells.

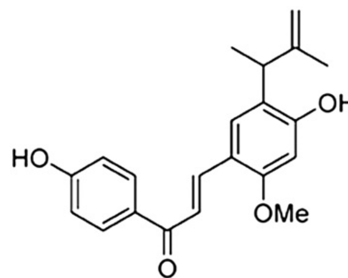


Fig. 1. Structure of Licochalcone E.

3.2. Lico-E protects DArgic SH-SY5Y cells against 6-OHDA-induced cell death

To examine whether Lico-E is cytoprotective against DArgic neurodegeneration, we used *in vitro* cytotoxicity model of PD in the human DArgic neuroblastoma cell line SH-SY5Y. Treatment with 100 μM 6-OHDA for 24 h evoked a significant increase in LDH activity in the cell culture medium, indicating cell death (LDH activity of 249%±31% of untreated control). Cells were treated with various doses of Lico-E for 1 h prior to exposure to 6-OHDA, and the degree of cell death was assessed after 24 h of 6-OHDA treatment. Lico-E alone did not have any cytotoxic effect at concentrations ranging from 0.1 to 2 μM and completely abolished the 6-OHDA-induced increase in LDH activity at 2 μM (Fig. 3A, LDH activity of 67%±2% of untreated control). Morphological analysis by inverted light microscopy (Fig. 3B) showed that the cells exposed to 100 μM 6-OHDA were shrunken, granular and round, suggesting cytotoxicity. Similar to the results from the LDH assay, pretreatment with different concentrations of Lico-E for 1 h revealed dose-dependent protective effects on the cell viability against the damage caused by 6-OHDA.

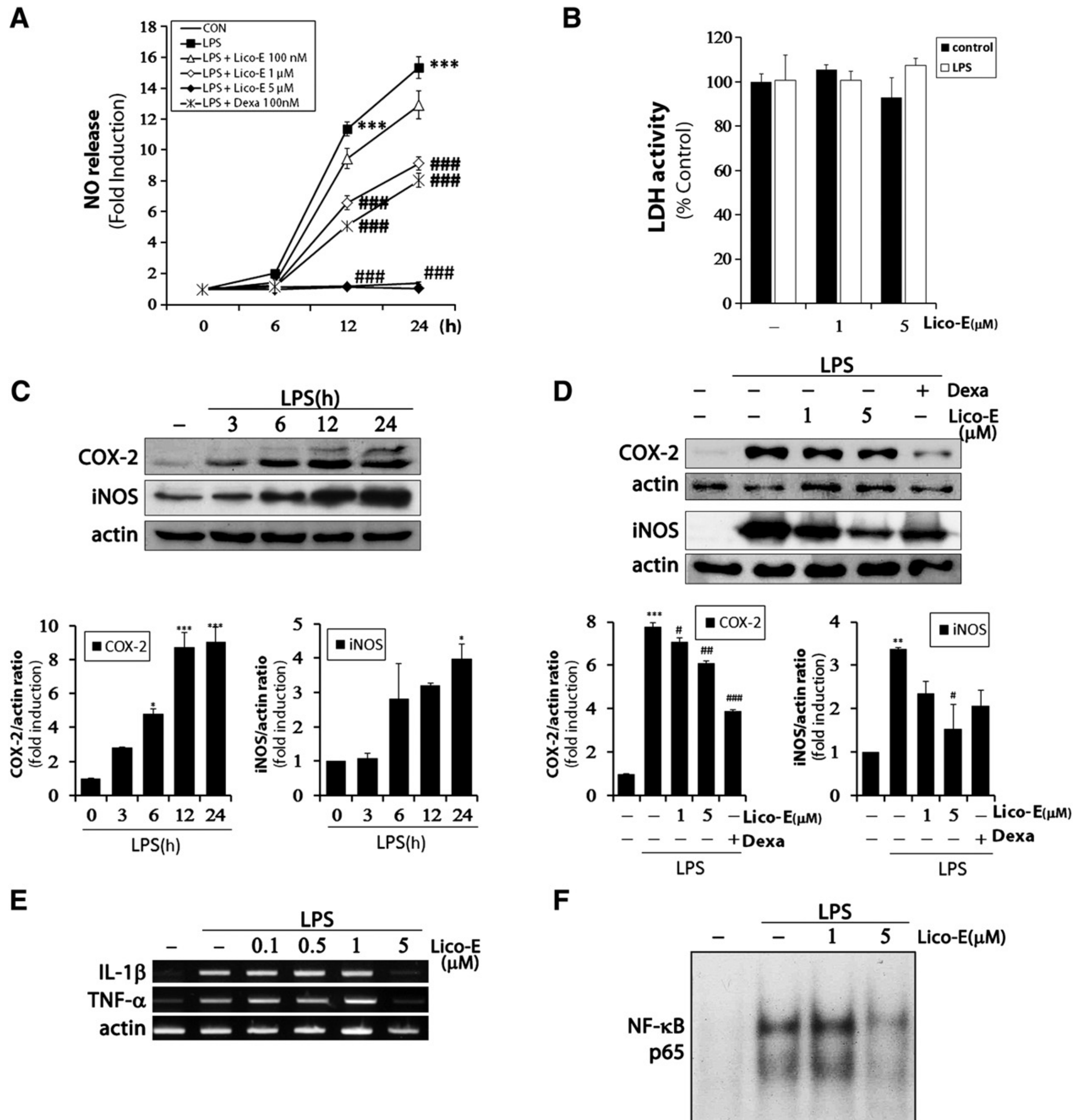


Fig. 2. Anti-inflammatory activity of Lico-E on LPS-induced stimulation of microglial BV2 cells. (A) BV2 cells were pretreated with 0.1, 1 or 5 μM Lico-E or 0.1 μM dexamethasone for 1 h and subsequently co-treated with 0.2 μg/ml LPS for the indicated durations (0, 6, 12 or 24 h). Released NO into the medium was assessed using the Griess reagent. NO generation was expressed as mean±S.E.M. of fold-induction of untreated control (n=4). Data are representative of at least three independent experiments; ***P<.001 versus respective untreated controls; ###P<.001 versus LPS-treated controls at respective indicated time point. (B) Cells were pretreated with Lico-E (1 or 5 μM) and subsequently co-treated with 0.2 μg/ml LPS for 24 h. Cytotoxicity was assessed by measuring LDH activity released into the medium. LDH activity was expressed as mean±S.E.M. as the percentage of untreated control (n=4). Data are representative of at least three independent experiments. (C and D) Cells were treated with 0.2 μg/ml LPS for the indicated durations (C; 0, 3, 6, 12 or 24 h) or pretreated with Lico-E or dexamethasone for 1 h and subsequently co-treated with 0.2 μg/ml LPS for 24 h (D). Equal amounts of protein (15 μg) were separated, and iNOS and COX-2 protein levels were analyzed by Western blotting analysis. β-Actin was used as a loading control. Data are representative of three or four experiments and were normalized to respective loading control and quantified by densitometric analysis. *P<.05, **P<.01 and ***P<.001 versus respective untreated control; #P<.05, ##P<.01 and ###P<.001 versus respective LPS-treated control. (E) Cells were pretreated with various concentrations (0.1, 0.5, 1 or 2 μM) of Lico-E for 1 h and subsequently co-treated with 0.2 μg/ml LPS for 1 h. mRNA levels of IL-1β and TNF-α were analyzed by RT-PCR. Data are representative of two or three experiments. (F) Transcriptional activity of NF-κB was assessed by EMSA. Cells were pretreated with 1 or 5 μM Lico-E for 1 h and subsequently co-treated with 0.2 μg/ml LPS for 24 h. The nuclear extracts (5 μg) were incubated with 1 μg of poly (dI-dC) and the [α-^{32}P]dCTP-labeled DNA probe. The protein/DNA complex was separated from the free probe in a 4.8% polyacrylamide gel. After electrophoresis, the gel was dried and autoradiographed. Data are representative of at least three independent experiments. Dexa, dexamethasone.

3.3. Lico-E induces nuclear translocation of Nrf2 and transcriptional activation of HO-1 and NQO1 enzyme

Recent studies have demonstrated that Nrf2 signaling plays a key role in regulating the cellular antioxidant response as well as in modulating the acute inflammatory response [31]. When activated, Nrf2 translocates from the cytosol to the nucleus and sequentially binds to a promoter region called the ARE, which is implicated in the regulation of downstream gene expression.

We asked whether the Nrf2 signaling pathway was activated and involved in the anti-inflammatory and cytoprotective activities of Lico-E. We first examined the effect of Lico-E on intracellular

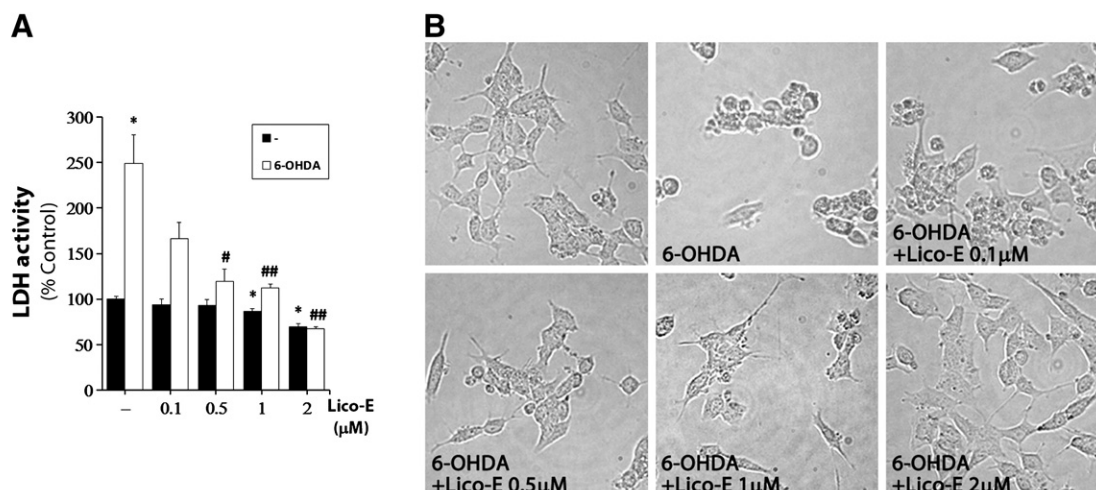


Fig. 3. Cytoprotective effect of Lico-E against 6-OHDA-induced DArgic SH-SY5Y cell death. SH-SY5Y cells were pretreated with different concentrations (0.1, 0.5, 1 or 2 μM) of Lico-E and subsequently co-treated with 100 μM 6-OHDA for 24 h. (A) Degrees of cell death were assessed by LDH activity released into the medium. LDH activity was expressed as mean±S.E.M. as the percentage of untreated control (n=4). Data are representative of at least three independent experiments; $^{*}P$<.05 versus untreated control; $^{\#}P$<.001 and $^{\#\#}P$<.01 versus 6-OHDA-treated control. (B) Representative photographs of morphological analysis. Cells were incubated with 6-OHDA and Lico-E as previously described, and their morphological changes were examined under inverted microscope.

localization of Nrf2 in HEK 293T, BV2 and SH-SY5Y cells. As shown in the immunofluorescence data in Figs. 4A, C and E, a relatively low level of Nrf2 protein was detected in the nuclear fraction of untreated cells, but the amount was up-regulated after treatment with Lico-E, suggesting translocation of Nrf2 from the cytoplasm to the nucleus in HEK 293T (Fig. 4A), BV2 (Fig. 4C) and SH-SY5Y (Fig. 4E) cells. Next, we performed reporter gene analysis for ARE to verify Lico-E-induced ARE activation [25]. Treatment with Lico-E induced significant increases in ARE-luciferase activity in those cell lines: 5.2±1.3-fold increase in HEK 293T cells after 6 h (Fig. 4B), 1.9±0.1-fold increase in BV2 cells after 3 h (Fig. 4D) and 1.8±0.2-fold increase in SH-SY5Y cells after 6 h (Fig. 4F).

As the activation of Nrf2 can lead to the up-regulation of genes important in the cellular antioxidant defense activity, we next asked whether protein expression levels of Nrf2 downstream antioxidant enzyme NQO1 and HO-1 were increased by Lico-E. Recent studies have shown that HO-1 plays a pivotal role in regulating inflammation [32]. As shown in Fig. 4G, Lico-E treatment significantly increased both HO-1 mRNA and protein levels in BV2 cells, suggesting that this enzyme contributes to the anti-inflammatory activity of Lico-E. In addition, the antioxidant enzyme NQO1 was also up-regulated by Lico-E in SH-SY5Y cells (Fig. 4H). All these findings suggest that Lico-E induces Nrf2 signaling and increases the levels of downstream enzyme proteins HO-1 and NQO1.

3.4. Nrf2-driven HO-1 and NQO1 induction mediates anti-inflammatory and cytoprotective activities of Lico-E, respectively

To confirm the contributions of HO-1 and NQO1 to the suppressive effects of Lico-E on LPS-induced inflammatory response and 6-OHDA cytotoxicity, respectively, we transiently transfected cells with Nrf2 siRNA and evaluated the effect of Lico-E on LPS-induced NO generation in BV2 cells and 6-OHDA-induced SH-SY5Y cell death. As shown in Fig. 5A and E, Nrf2 siRNA inhibited the protein expression of Nrf2 in both cell lines and suppressed Lico-E-induced up-regulation of HO-1 in BV2 cells (Fig. 5B) and of NQO1 in SH-SY5Y cells (Fig. 5F). We further examined whether the anti-inflammatory effect of Lico-E was abolished by siRNA knockdown of Nrf2. As expected, Lico-E-induced attenuation of LPS-induced inflammatory responses in BV2 cells was abolished by silencing Nrf2 expression (Fig. 5C). Lico-E-induced cytoprotection against 6-OHDA was also abolished by silencing Nrf2 expression with specific siRNA in SH-SY5Y cells (Fig. 5G). In addition, the anti-inflammatory and cytoprotective effects of Lico-E were attenuated by the specific HO-1 inhibitor ZnPP and the NQO1 inhibitor dicoumarol, respectively (Fig. 5D and H). These data suggest a crucial role for the transcriptional activator Nrf2 and its downstream enzymes in the anti-inflammatory and cytoprotective potential of Lico-E.

3.5. Lico-E attenuates MPTP-induced nigrostriatal DArgic neurodegeneration

We next examined whether the potential of Lico-E to regulate inflammatory responses and cytotoxicity is beneficial in an experimental animal model of PD. Among several animal models of PD, MPTP can reproduce characteristic pathology of PD when administered to mice, i.e., degeneration of nigrostriatal DArgic neurons and the activation of microglia in the vicinity of DArgic neurons [33,34]. An increase in the generation of ROS is believed to play a crucial role in MPTP toxicity, and there is much evidence showing Nrf2-mediated neuronal protection against MPTP [35]. Ten milligrams per kilogram Lico-E in 10% ethanol in saline was orally administered for 5 days, and the cytoprotective effect of Lico-E against MPTP-induced DArgic neurodegeneration was evaluated. We evaluated the expression level of TH in SNpc and striatum of the brain, which is the enzyme responsible for DArgic neurons as markers of DArgic toxicity. Ten percent ethanol in saline did not cause significant changes in TH expression level in both brain regions (Fig. 6A) as well as any differences in body weight or behavior compared with saline-treated group. Fig. 6B showed a significant decrease in TH protein level in both striatum and SNpc of MPTP-treated mice. Lico-E treatment attenuated MPTP-induced TH loss in both regions compared to MPTP-treated group. These results suggest that Lico-E protects DArgic neurons against MPTP-induced degeneration *in vivo*.

3.6. Lico-E up-regulates HO-1 and NQO1 expression in the substantia nigra of mice brain

Because Lico-E activated the Nrf2 pathway *in vitro* and up-regulation of HO-1 and NQO1 was important for the anti-inflammatory and cytoprotective effects of Lico-E, we next evaluated the effect of Lico-E on the expression of HO-1 and NQO1 *in*

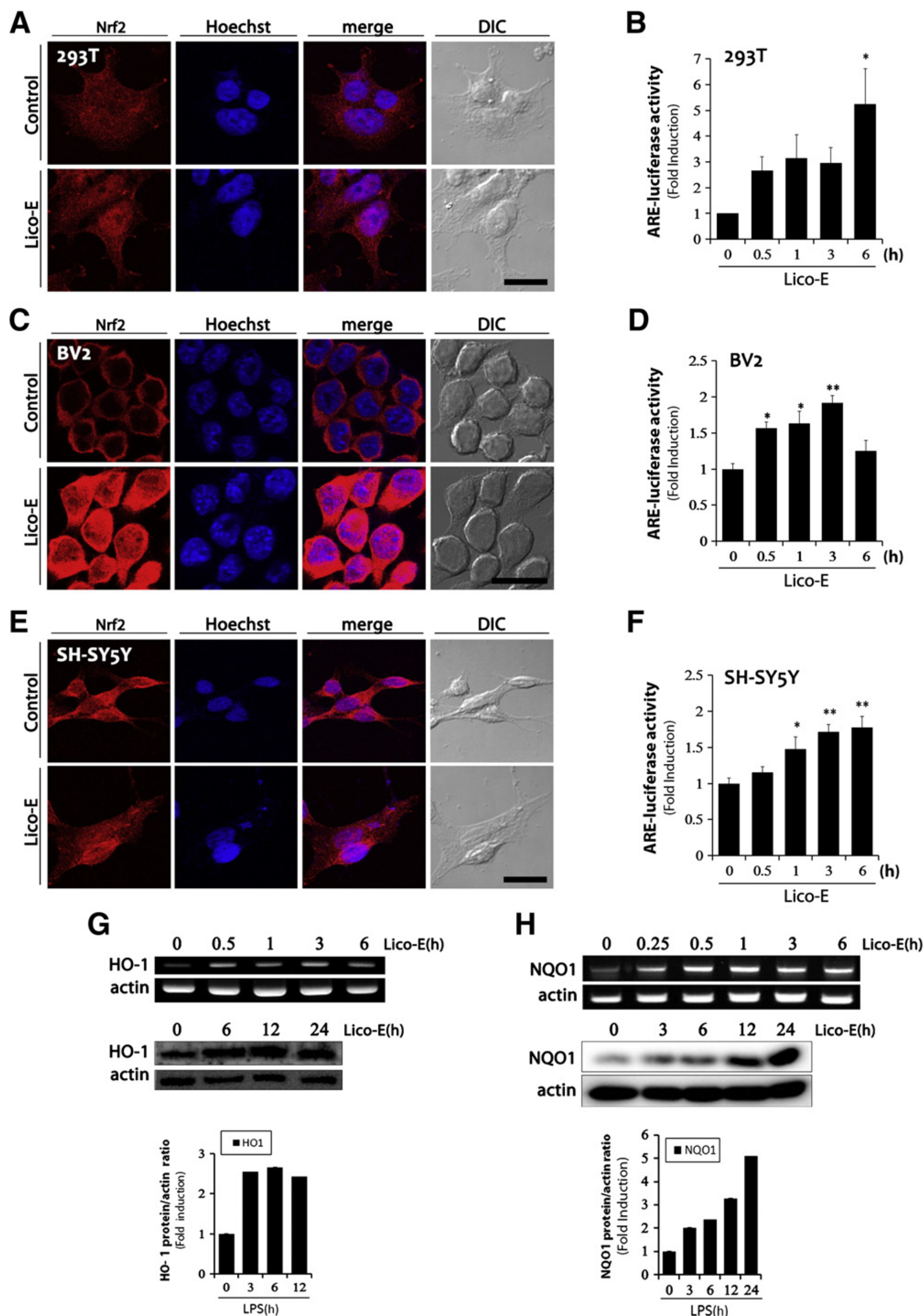
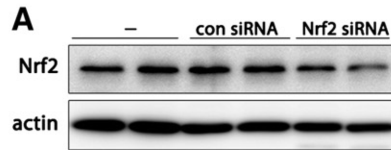
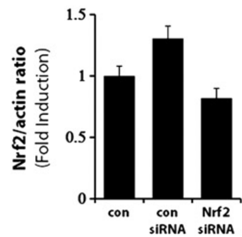


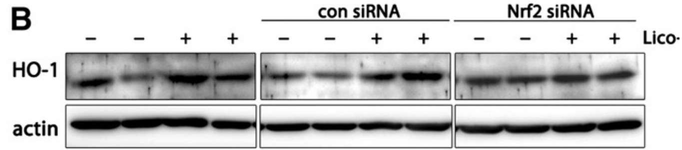
Fig. 4. Induction of the Nrf2-driven antioxidant and anti-inflammatory enzyme expression by Lico-E. (A, C and E) HEK 293T cells (A) and BV2 cells (C) were treated with 5 μM Lico-E for 6 h, and SH-SY5Y cells (E) were treated with 2 μM Lico-E for 6 h. The levels of Nrf2 protein in cytosolic and nuclear fractions were analyzed by immunofluorescent confocal microscopic analysis (red fluorescence). Hoechst staining (blue fluorescence) represents the nuclei. (B, D, and F) HEK 293T (B), BV2 (D) and SH-SY5Y (F) cells were treated with 5 (B and D) and 2 μM (F) Lico-E for the indicated time (0, 0.5, 1, 3 or 6 h), respectively, and ARE-luciferase activity was measured. Luciferase activity was normalized to the renilla luciferase activity and presented in relative luciferase units. Data are representative of at least two independent experiments and expressed as mean±S.E.M. of fold-induction relative to untreated cells (at time zero) (n=4); *P<.05 and **P<.01 versus untreated control. (G and H) BV2 cells (G) were treated with 5 μM Lico-E for the indicated durations, and mRNA (upper panel) and protein levels (lower panel) of HO-1 were analyzed by RT-PCR and Western blotting, respectively. SH-SY5Y cells (H) were treated with 2 μM Lico-E for the indicated durations, and mRNA (upper panel) and protein levels (lower panel) of NQO1 were analyzed by RT-PCR and Western blotting. For Western blotting, equal amounts of protein (20 μg) were separated and incubated with HO-1 (G) and NQO1 (H) antibodies, respectively. β-Actin was used as a loading control. Data are representative of two or three experiments, and Western blotting was normalized to respective loading control and quantified by densitometric analysis. Scale bar, 20 μm.

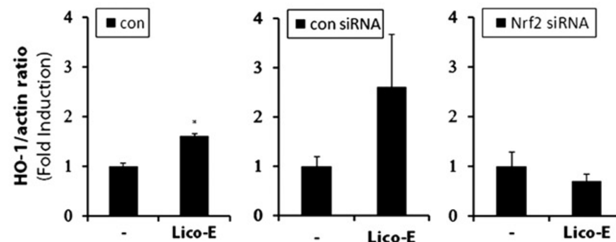
vivo, especially in the nigrostriatal brain region. The expression of Nrf2 protein was not changed by Lico-E administration (Fig. 7A). But as shown in Fig. 7B, HO-1 was up-regulated in the SNpc of Lico-E-treated mice (1.4±0.1-fold of control mice in SNpc). The expression of NQO1 protein was significantly increased by Lico-E in both SNpc and striatum (Fig. 7C; 3.6±0.2-fold and 1.8±0.2-fold of control mice in SNpc and striatum, respectively). Collectively, these data showed that Lico-E had the potential to induce Nrf2-

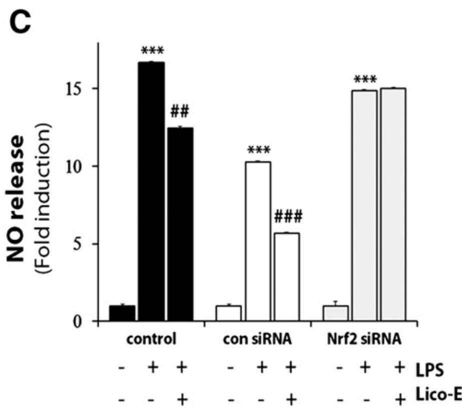
BV2>

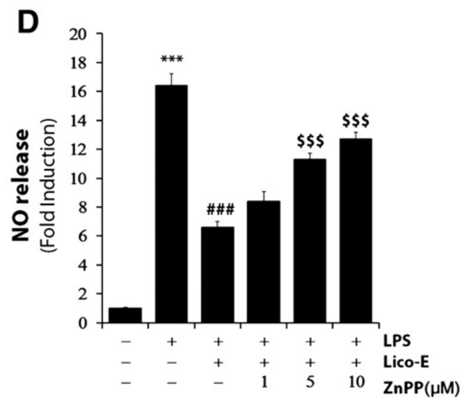




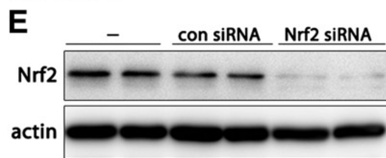


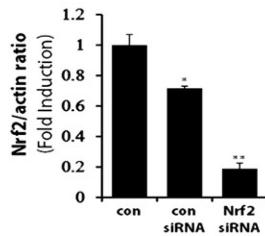


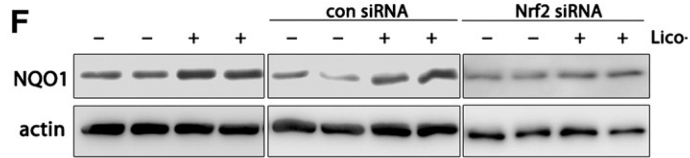


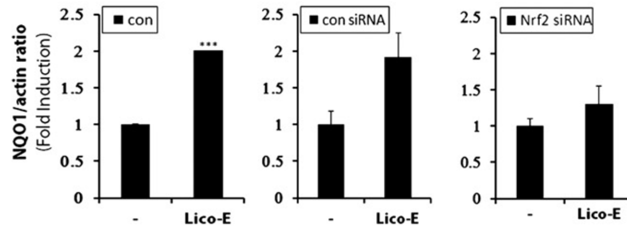


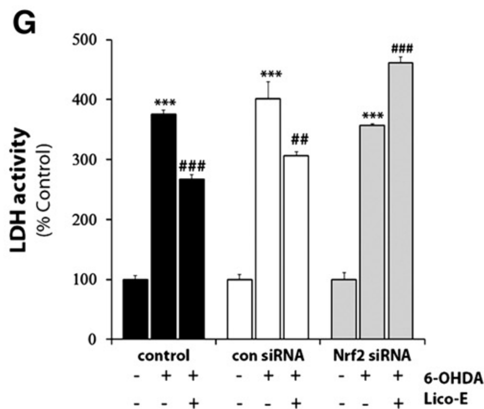
SH-SY5Y>

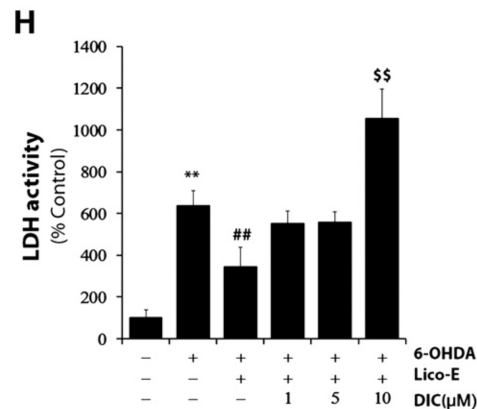












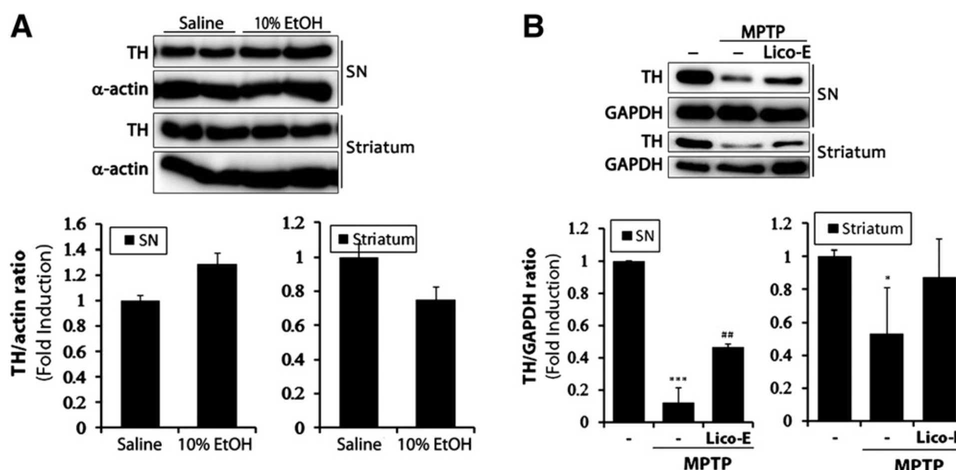


Fig. 6. Effects of Lico-E on the levels of TH protein in MPTP-treated mouse brain. (A) Mice were orally administered saline or saline including 10% ethanol for 5 days, and then TH protein levels in SNpc and striatum were measured by Western blotting analysis. α-Actin was used as a loading control. (B) Mice were orally administered either Lico-E (10 mg/kg) or 10% ethanol in saline (control) for 5 days and then injected with MPTP (20 mg/kg intraperitoneally, four doses every 2 h in 1 day). After 7 days, brain was removed, and TH protein levels in SNpc and striatum were measured by Western blotting analysis against TH antibody. GAPDH was used as a loading control. Data are representative of two or three experiments, and were quantified by densitometric analysis. $^{*}P<.05$ and $^{***}P<.001$ versus control group; $^{\#\#}P<.01$ versus MPTP-alone-treated group. SN, substantia nigra.

downstream HO-1 and NQO1 protein expression in the nigrostriatal region of the mouse brain.

4. Discussion

The present study demonstrates that (a) Lico-E obtained from the roots of *G. inflata* B. attenuates LPS-induced inflammatory responses in microglial BV2 cells and protects DArgic SH-SY5Y cells from 6-OHDA cytotoxicity, (b) Lico-E has neuroprotective effects against MPTP-induced nigrostriatal DArgic neurodegeneration in mice and (c) Lico-E activates the Nrf2 pathway and up-regulates downstream antioxidant enzyme expressions both *in vitro* and *in vivo*.

Retrochalcones including licochalcone A–D (Lico A–D), phenolic constituents of the licorice species, are suggested to have antioxidant and oxygen-scavenging activities and to protect biological systems against various oxidative stresses [17]. Studies have also shown that chalcone derivatives exhibit potent anti-inflammatory activity [36]. For example, Lico-A acts as a potent inhibitor of proinflammatory responses, including the zymosan-induced oxidative burst of granulocytes, the LPS-induced PGE2 release by dermal fibroblasts, the *N*-formyl-MET-LEU-PHE-induced LTB4 release by granulocytes and the LPS-induced IL-6/TNFα secretion by monocyte-derived dendritic cells [16]. Furusawa et al. [37] suggested that the anti-inflammatory activity of Lico-A could be mediated by the inhibition of NF-κB transcriptional activation. Other *G.-inflata*-derived chalcones Lico-B and -D are also known to inhibit phosphorylation of NF-κB p65 in LPS signaling pathway [38]. Although it is unclear which mechanism contributes to the anti-inflammatory activity of chalcone derivatives, regulation of intracellular and extracellular ROS generation could play an important role. In fact, ROS released by microglia contribute to the elimination of pathogens and also act as second messengers that activate NF-κB signaling, which results in further expression of proinflammatory cytokines [39].

In the present study, we show the potential involvement of Nrf2 signaling in both the anti-inflammatory and the antioxidant activities elicited by Lico-E. Lico-E induces nuclear translocation of Nrf2 (Fig. 4A, C and E), ARE-promoter activation (Fig. 4B, D and F) and transcription of cytoprotective NQO1 and HO-1 (Fig. 4G and H) *in vitro* and *in vivo* (Fig. 7). Moreover, the anti-inflammatory and cytoprotective effects of Lico-E are attenuated in Nrf2-silenced cells using Nrf2 siRNA (Fig. 5C and G) as well as in the presence of specific inhibitors of HO-1 and NQO1, respectively (Fig. 5D and H). Nrf2 is a kind of redox-sensitive protein transcription factor that regulates the expression of a set of genes with ARE in their promoters such as NQO1 and HO-1; Nrf2 is therefore essential in the *de novo* regulation of various antioxidant enzymes. From this perspective, chemicals activating Nrf2 signaling could be especially desirable for preventing free-radical-mediated cellular injuries as well as for protection against cytotoxicity and proinflammatory insults. Among the compounds that are able to control the transcriptional activity of Nrf2, dietary polyphenols such as epigallocatechin-3-gallate [40], resveratrol [41] and isoorientin [42] have received much attention for their ability to induce Nrf2 signaling and maximize the intrinsic antioxidant potential. Structurally, Lico-E belongs to a class of polyphenols (Fig. 1). The difference between the known dietary polyphenols with Nrf2

Fig. 5. Attenuation of anti-inflammatory and cytoprotective activities of Lico-E by Nrf2 knockdown and by specific inhibitors of HO-1 and NQO1. BV2 (A–C) and SH-SY5Y (E–G) cells were transiently transfected with Nrf2 siRNA (100 pmol/1×10^6 cells) or control siRNA (100 pmol/1×10^6 cells) for 24 h. Nrf2 (A and E) protein levels were measured by Western blotting analysis. Twenty-four hours after Nrf2 siRNA transfection, BV2 cells (B) were treated with 5 μM Lico-E for an additional 24 h, and the expression level of HO-1 was measured. The effect of Lico-E (2 μM) on NQO1 expression was also examined in control-, control siRNA-transfected-, and Nrf2-siRNA-transfected SH-SY5Y cells (F). β-Actin was used as a loading control. (C and G) After Nrf2 siRNA transfection, BV2 cells (C) were treated with 5 μM Lico-E for 1 h and subsequently co-treated with 0.2 μg/ml LPS for an additional 24 h. LPS-induced NO generation was measured and expressed as mean±S.E.M. as fold-induction of untreated control (n=4). Nrf2-siRNA-transfected SH-SY5Y cells (G) were pretreated with 2 μM Lico-E and subsequently co-treated with 100 μM 6-OHDA for 24 h. Degrees of cell death were assessed by LDH activity in the medium. LDH activity is expressed as mean±S.E.M. in percentage of untreated control (n=4); $^{***}P<.001$ versus untreated respective control; $^{\#\#}P<.01$ and $^{\#\#\#}P<.001$ versus LPS-treated (C) and 6-OHDA-treated (G) control cells, respectively. (D and H) BV2 cells (D) were pretreated with 5 μM Lico-E for 1 h in the presence or absence of HO-1 inhibitor ZnPP (1, 5 or 10 μM) and subsequently co-treated with 0.2 μg/ml LPS for an additional 24 h. NO generation was expressed as mean±S.E.M. as fold-induction of untreated control (n=4). SH-SY5Y cells (H) were pretreated with 2 μM Lico-E for 1 h in the presence or absence of NQO1 inhibitor dicoumarol (1, 5 or 10 μM) and subsequently co-treated with 100 μM 6-OHDA for 24 h. LDH activity was expressed as mean±S.E.M. in percentage of untreated control (n=4); $^{**}P<.01$ and $^{***}P<.001$ versus untreated control; $^{\#\#}P<.01$ and $^{\#\#\#}P<.001$ versus LPS-treated (D) and 6-OHDA-treated (H) control cells, respectively; $^{\$\$}P<.01$ and $^{\$\$\$}P<.001$ versus Lico-E+LPS-treated (D) and Lico-E+6-OHDA-treated (H) cells, respectively. All data are representative of at least two independent experiments. DIC, dicoumarol.

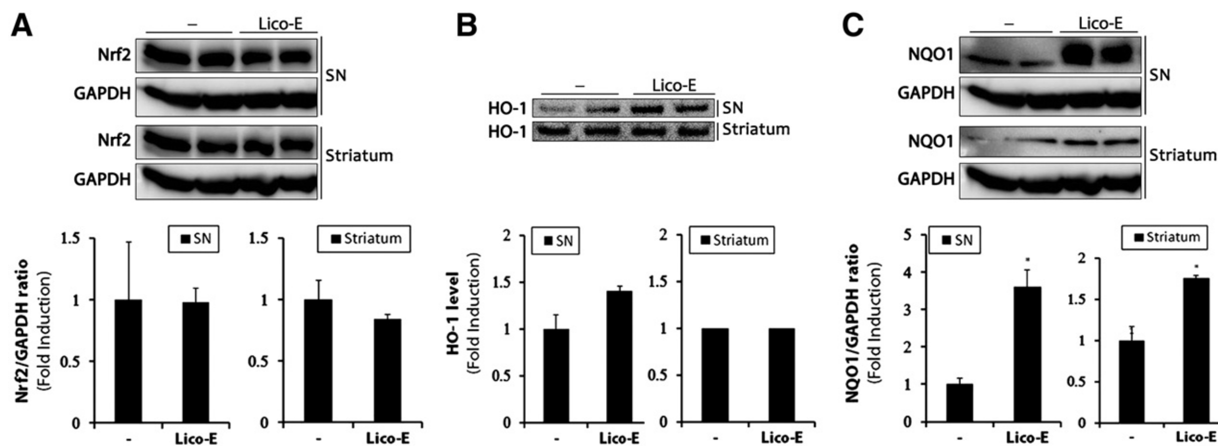


Fig. 7. Up-regulation of HO-1 and NQO1 in the substantia nigra (SNpc) and striatum of Lico-E-treated mice. Mice were orally administered Lico-E (10 mg/kg) or saline including 10% ethanol (control) for 5 days, and SNpc and striatum were removed. Equal amounts of protein (10 μg) were separated, and the levels of Nrf2 (A), HO-1 (B) and NQO1 (C) in SNpc and striatal sections were analyzed by Western blot analysis. GAPDH was used as a loading control. Data are representative of two or three experiments, and were quantified by densitometric analysis. *$P<.05$ versus control.

modulating activity and Lico-E is that Lico-E also has an α,β-unsaturated carbonyl moiety. Because the α,β-unsaturated carbonyl group is electrophilic and can function as a Michael reaction acceptor, Lico-E readily interacts with critical cellular nucleophiles, such as cysteine thiol groups in proteins, to form covalent adducts. Also, the α,β-unsaturated carbonyl group interferes with Keap1–Nrf2 binding and therefore causes activation of Nrf2 signaling [43]. This may result in alterations of cellular redox status and/or functions of target proteins, and Michael reaction acceptors are known to be strong activators of the Nrf2 signaling pathway [44]. In fact, some flavonoids lacking the α,β-unsaturated carbonyl group are reported to show weak or no inhibitory effect on LPS-induced inflammatory responses [45].

Nrf2 is present in most cell types of the brain including DArgic neurons and glial cells, where it participates in redox homeostasis by regulating antioxidant gene expression. Recent evidence suggests that Nrf2 signaling is relevant in the neuroprotection of DArgic neurons. Aberrant or insufficient Nrf2 signaling is found in neurodegenerative conditions [46]; for example, DArgic neurons of Nrf2-deficient mice are more susceptible to MPTP or 6-OHDA-induced degeneration [47,48], and overexpression of Nrf2 can rescue neurons from various neurotoxic attacks [49]. Despite the relevance of Nrf2-activating compounds for the neuroprotection of DArgic neurons, the usefulness of such compounds in disease is unclear due to the lack of information regarding their ability to enter the central nervous system. In the search for a compound that might activate Nrf2 signaling in the brain, compounds that easily pass the blood brain barrier (BBB) and can be taken up by neuronal or glial cells can be developed as drugs. Although we have not directly evaluated whether Lico-E traverses the BBB, our results show that oral administration of Lico-E activates the Nrf2-ARE pathway. This is evidenced by (a) the cytoprotective activity of Lico-E not only in the *in vitro* cell culture system but also in the *in vivo* MPTP animal model (Fig. 6B) and (b) the up-regulation of HO-1 and NQO1 in the brain of Lico-E-treated mice (Fig. 7). Generally, hydrophobic character is the most important determinant of a drug's ability to cross the BBB. Hansch and colleagues [50] have suggested that a logP around 2 results in maximal BBB penetration. Lico-E is rather lipophilic with a logP value of 4.75 so it is not unreasonable that Lico-E can penetrate BBB. Of course, one cannot eliminate the possibility that Lico-E is metabolized before entering the brain and that the metabolite penetrates BBB and activates Nrf2 signaling. Further experiments are in progress to identify the exact mechanism of Lico-E-induced activation of Nrf2 signaling in the brain.

In conclusion, the present study shows that Lico-E protects DArgic SH-SY5Y cells from 6-OHDA and attenuates LPS-induced inflammatory responses in microglial BV2 cells by a mechanism involving Nrf2 activation and up-regulation of the expression of its downstream antioxidant enzymes. Lico-E rescues nigrostriatal DArgic neurons from MPTP toxicity in mice. According to our results, the recently identified *G.-inflata*-derived chalcone Lico-E could be protective against DArgic neurodegeneration evoked by oxidative insults and inflammatory responses and could be readily available for PD therapeutics.

References

[1] Dawson T, Dawson V. Molecular pathways of neurodegeneration in Parkinson's disease. Science 2003;302:819–22.
[2] Tansey M, Frank-Cannon T, McCoy M, Lee J, Martinez T, McAlpine F, et al. Neuroinflammation in Parkinson's disease: is there sufficient evidence for mechanism-based interventional therapy? Front Biosci 2008;13:709–17.
[3] No H, Bang Y, Lim J, Kim S, Choi H, Choi H. Involvement of induction and mitochondrial targeting of orphan nuclear receptor Nur77 in 6-OHDA-induced SH-SY5Y cell death. Neurochem Int 2010;56:620–6.
[4] Offen D, Ziv I, Sternin H, Melamed E, Hochman A. Prevention of dopamine-induced cell death by thiol antioxidants: possible implications for treatment of Parkinson's disease. Exp Neurol 1996;141:32–9.
[5] Hastings T, Lewis D, Zigmond M. Reactive dopamine metabolites and neurotoxicity: implications for Parkinson's disease. Adv Exp Med Biol 1996;387:97–106.
[6] Choi HJ, Kim SW, Lee SY, Hwang O. Dopamine-dependent cytotoxicity of tetrahydrobiopterin: a possible mechanism for selective neurodegeneration in Parkinson's disease. J Neurochem 2003;86:143–52.
[7] Chae S, Bang Y, Kim K, Lee K, Kang B, Kim E, et al. Role of cyclooxygenase-2 in tetrahydrobiopterin-induced dopamine oxidation. Biochem Biophys Res Commun 2007;359:735–41.
[8] Müller T, Blum-Degen D, Przuntek H, Kuhn W. Interleukin-6 levels in cerebrospinal fluid inversely correlate to severity of Parkinson's disease. Acta Neurol Scand 1998;98:142–4.
[9] Whitton P. Inflammation as a causative factor in the aetiology of Parkinson's disease. Br J Pharmacol 2007;150:963–76.
[10] Knott C, Stern G, Wilkin G. Inflammatory regulators in Parkinson's disease: iNOS, lipocortin-1, and cyclooxygenases-1 and -2. Mol Cell Neurosci 2000;16:724–39.
[11] Nioi P, Hayes J. Contribution of NAD(P)H:quinone oxidoreductase 1 to protection against carcinogenesis, and regulation of its gene by the Nrf2 basic-region leucine zipper and the arylhydrocarbon receptor basic helix–loop–helix transcription factors. Mutat Res 2004;555:149–71.
[12] Talalay P, Dinkova-Kostova A. Role of nicotinamide quinone oxidoreductase 1 (NQO1) in protection against toxicity of electrophiles and reactive oxygen intermediates. Methods Enzymol 2004;382:355–64.
[13] Owuor E, Kong A. Antioxidants and oxidants regulated signal transduction pathways. Biochem Pharmacol 2002;64:765–70.

[14] Prawan A, Kundu J, Surh Y. Molecular basis of heme oxygenase-1 induction: implications for chemoprevention and chemoprotection. Antioxid Redox Signal 2005;7:1688–703.
[15] Lehmann J, Listopad J, Rentzsch C, Igney F, von Bonin A, Hennekes H, et al. Dimethylfumarate induces immunosuppression via glutathione depletion and subsequent induction of heme oxygenase 1. J Invest Dermatol 2007;127:835–45.
[16] Kolbe L, Immeyer J, Batzer J, Wensorra U, tom Dieck K, Mundt C, et al. Anti-inflammatory efficacy of licochalcone A: correlation of clinical potency and in vitro effects. Arch Dermatol Res 2006;298:23–30.
[17] Haraguchi H, Ishikawa H, Mizutani K, Tamura Y, Kinoshita T. Antioxidative and superoxide scavenging activities of retrochalcones in *Glycyrrhiza inflata*. Bioorg Med Chem 1998;6:339–47.
[18] Yoon G, Kang B, Cheon S. Topoisomerase I inhibition and cytotoxicity of licochalcones A and E from *Glycyrrhiza inflata*. Arch Pharm Res 2007;30:313–6.
[19] Saitoh T, Shibata S. New type chalcones from licorice root. Tetrahedron Lett 1975;16:4461–2.
[20] Yoon G, Jung Y, Cheon S. Cytotoxic allyl retrochalcone from the roots of *Glycyrrhiza inflata*. Chem Pharm Bull (Tokyo) 2005;53:694–5.
[21] Cho Y, Lee S, Yoon G, Kim H, Na J, Choi H, et al. Licochalcone E reduces chronic allergic contact dermatitis and inhibits IL-12p40 production through down-regulation of NF-kappaB. Int Immunopharmacol 2010;10:1119–26.
[22] Foresti R, Hoque M, Monti D, Green C, Motterlini R. Differential activation of heme oxygenase-1 by chalcones and rosolic acid in endothelial cells. J Pharmacol Exp Ther 2005;312:686–93.
[23] Perjési P, Ember I, Bozak R, Nádasi E, Rozmer Z, Varjas T, et al. Effect of the chalcone analog E,E-bis(2-hydroxybenzylidene) acetone on the 7,12-dimethylbenz[a]anthracene-induced Ha-ras gene activity in vivo. In Vivo 2006;20: 141–6.
[24] Lim J, Kim K, Kim S, Hwang O, Choi H. Bromocriptine activates NQO1 via Nrf2-PI3K/Akt signaling: novel cytoprotective mechanism against oxidative damage. Pharmacol Res 2008;57:325–31.
[25] Kang K, Cho I, Lee C, Kim S. Essential role of phosphatidylinositol 3-kinase-dependent CCAAT/enhancer binding protein beta activation in the induction of glutathione S-transferase by oltipraz. J Natl Cancer Inst 2003;95:53–66.
[26] Cui Y, Ao M, Li W, Hu J, Yu L. Anti-inflammatory activity of licochalcone A isolated from *Glycyrrhiza inflata*. Z Naturforsch C 2008;63:361–5.
[27] Thomas D, Francescutti-Verbeem D, Kuhn D. Gene expression profile of activated microglia under conditions associated with dopamine neuronal damage. FASEB J 2006;20:515–7.
[28] Alcaraz M, Vicente A, Araico A, Dominguez J, Terencio M, Ferrándiz M. Role of nuclear factor-kappaB and heme oxygenase-1 in the mechanism of action of an anti-inflammatory chalcone derivative in RAW 264.7 cells. Br J Pharmacol 2004;142:1191–9.
[29] Moncada S, Higgs A. The L-arginine-nitric oxide pathway. N Engl J Med 1993;329: 2002–12.
[30] Radomski M, Palmer R, Moncada S. Glucocorticoids inhibit the expression of an inducible, but not the constitutive, nitric oxide synthase in vascular endothelial cells. Proc Natl Acad Sci U S A 1990;87:10043–7.
[31] New type chalcones from licorice root. Tetrahedron Letters. 1975;16:4461–2.
[32] Syapin P. Regulation of haeme oxygenase-1 for treatment of neuroinflammation and brain disorders. Br J Pharmacol 2008;155:623–40.
[33] Członkowska A, Kohutnicka M, Kurkowska-Jastrzebska I, Członkowski A. Microglial reaction in MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) induced Parkinson's disease mice model. Neurodegeneration 1996; 5:137–43.
[34] Jackson-Lewis V, Smeyne R. MPTP and SNpc DA neuronal vulnerability: role of dopamine, superoxide and nitric oxide in neurotoxicity. Mini Rev Neurotox Res 2005;7:193–202.
[35] Chen P, Vargas M, Pani A, Smeyne R, Johnson D, Kan Y, et al. Nrf2-mediated neuroprotection in the MPTP mouse model of Parkinson's disease: critical role for the astrocyte. Proc Natl Acad Sci U S A 2009;106:2933–8.
[36] Herencia F, Ferrándiz M, Ubeda A, Domínguez J, Charris J, Lobo G, et al. Synthesis and anti-inflammatory activity of chalcone derivatives. Bioorg Med Chem Lett 1998;8:1169–74.
[37] Furusawa J, Funakoshi-Tago M, Tago K, Mashino T, Inoue H, Sonoda Y, et al. Licochalcone A significantly suppresses LPS signaling pathway through the inhibition of NF-kappaB p65 phosphorylation at serine 276. Cell Signal 2009;21: 778–85.
[38] Furusawa J, Funakoshi-Tago M, Mashino T, Tago K, Inoue H, Sonoda Y, et al. *Glycyrrhiza inflata*-derived chalcones, licochalcone A, licochalcone B and licochalcone D, inhibit phosphorylation of NF-kappaB p65 in LPS signaling pathway. Int Immunopharmacol 2009;9:499–507.
[39] Pawate S, Shen Q, Fan F, Bhat N. Redox regulation of glial inflammatory response to lipopolysaccharide and interferongamma. J Neurosci Res 2004;77: 540–51.
[40] Wu C, Hsu M, Hsieh C, Lin J, Lai P, Wung B. Upregulation of heme oxygenase-1 by Epigallocatechin-3-gallate via the phosphatidylinositol 3-kinase/Akt and ERK pathways. Life Sci 2006;78:2889–97.
[41] Juan M, González-Pons E, Munuera T, Ballester J, Rodríguez-Gil J, Planas J. *trans*-Resveratrol, a natural antioxidant from grapes, increases sperm output in healthy rats. J Nutr 2005;135:757–60.
[42] Lim J, Park H, Choi J, Lee I, Choi H. Isoorientin induces Nrf2 pathway-driven antioxidant response through phosphatidylinositol 3-kinase signaling. Arch Pharm Res 2007;30:1590–8.
[43] Wu R, Hayashi T, Cottam H, Jin G, Yao S, Wu C, et al. Nrf2 responses and the therapeutic selectivity of electrophilic compounds in chronic lymphocytic leukemia. Proc Natl Acad Sci U S A 2010;107:7479–84.
[44] Dinkova-Kostova A, Massiah M, Bozak R, Hicks R, Talalay P. Potency of Michael reaction acceptors as inducers of enzymes that protect against carcinogenesis depends on their reactivity with sulfhydryl groups. Proc Natl Acad Sci U S A 2001;98:3404–9.
[45] Liu Y, Hsieh C, Wu C, Wung B. Chalcone inhibits the activation of NF-kappaB and STAT3 in endothelial cells via endogenous electrophile. Life Sci 2007;80: 1420–30.
[46] Ramsey C, Glass C, Montgomery M, Lindl K, Ritson G, Chia L, et al. Expression of Nrf2 in neurodegenerative diseases. J Neuropathol Exp Neurol 2007;66:75–85.
[47] Burton N, Kensler T, Guilarte T. In vivo modulation of the Parkinsonian phenotype by Nrf2. Neurotoxicology 2006;27:1094–100.
[48] Jakel R, Townsend J, Kraft A, Johnson J. Nrf2-mediated protection against 6-hydroxydopamine. Brain Res 2007;1144:192–201.
[49] Calkins M, Jakel R, Johnson D, Chan K, Kan Y, Johnson J. Protection from mitochondrial complex II inhibition in vitro and in vivo by Nrf2-mediated transcription. Proc Natl Acad Sci U S A 2005;102:244–9.
[50] Hansch C, Björkroth J, Leo A. Hydrophobicity and central nervous system agents: on the principle of minimal hydrophobicity in drug design. J Pharm Sci 1987;76: 663–87.