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3,4-Dihydroxybenzalacetone Protects Against Parkinson's Disease-Related Neurotoxin 6-OHDA Through Akt/Nrf2/Glutathione Pathway

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ABSTRACT

Oxidative stress is implicated in the pathogenesis of various neurodegenerative diseases including Parkinson's disease (PD). 3,4-Dihydroxybenzalacetone (DBL) is a small catechol-containing compound isolated from Chaga (*Inonotus obliquus* [persoon] Pilat), and has been reported to have beneficial bioactivities, including antioxidative, anti-inflammatory, and anti-tumorigenic activities, with a relatively low toxicity to normal cells. We, therefore, investigated the neuroprotective activity of DBL against the PD-related neurotoxin 6-hydroxydopamine (6-OHDA). Pretreatment of human neuroblastoma SH-SY5Y cells with DBL, but not with another Chaga-derived catechol-containing compound, caffeic acid, dose-dependently improved the survival of 6-OHDA-treated cells. Although DBL did not reduce 6-OHDA-induced reactive oxygen species in the cell-free system, it promoted the translocation of Nrf2 to the nucleus, activated the transcription of Nrf2-dependent antioxidative genes, and increased glutathione synthesis in the cells. Buthionine sulfoximine, an inhibitor of glutathione synthesis, but not Sn-mesoporphyrin IX, a heme oxygenase-1 inhibitor, or dicoumarol, an NAD(P)H:quinone oxidoreductase 1 inhibitor, abolished the protective effect of DBL against 6-OHDA. Furthermore, DBL activated stress-associated kinases such as Akt, ERK, and p38 MAPK, and PI3K or Akt inhibitors, but not ERK, p38, or JNK inhibitors, diminished DBL-induced glutathione synthesis and protection against 6-OHDA. These results suggest that DBL activates the Nrf2/glutathione pathway through PI3K/Akt, and improves survival of SH-SY5Y cells against 6-OHDA toxicity. J. Cell. Biochem. 115: 151–160, 2014. © 2013 Wiley Periodicals, Inc.

KEY WORDS: NEURONAL DEATH; GLUTATHIONE; PARKINSON'S DISEASE

Parkinson's disease (PD) is a progressive neurodegenerative disease pathologically characterized by the selective loss of the nigrostriatal dopaminergic neurons and the presence of protein aggregates, known as Lewy bodies [for a review, see Dauer and Przedborski, 2003]. Several neurotoxins, including 6-hydroxydopamine (6-OHDA) and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), or the mutation of genes, such as the one encoding α-synuclein, have been used to model PD in vivo and in vitro. Most of these approaches are associated with mitochondrial damage and/or enhanced oxidative stress. 6-OHDA is a dopamine (DA) derivative which generates high levels of reactive oxygen species (ROS) by extracellular auto-oxidation and by intracellular accumulation

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through the dopamine transporter (DAT) [Dauer and Przedborski, 2003; Hanrott et al., 2006]. In the cells, enhanced ROS inactivates biological macromolecules, perturbs organelle function, and induces cell death [Dauer and Przedborski, 2003; Hanrott et al., 2006]. In contrast, MPTP is a highly lipophilic compound and crosses the blood–brain barrier. MPTP is converted to the active form, 1-methyl-4-phenylpyridinium (MPP^+) by astrocytes in the brain, and taken up by dopaminergic neurons again through the DAT. MPP^+ inhibits the activity of mitochondrial complex I and generates ROS. These lines of evidence suggest that it is critical to identify small anti-oxidants with lower toxicity that can cross the blood–brain barrier.

Inonotus obliquus (persoon) Pilat (Chaga in Russia and Kabanoa-natake, or Chaga in Japan) is an overgrowth of fungus that is found mainly in the trunk of birch trees. Chaga has been used for the prophylaxis and treatment of gastric disorders and cancers in Russia [Shashkina et al., 2006]. Recently, several investigators have published reports on the beneficial bioactivities of Chaga, such as antioxidative [Park et al., 2004], anti-inflammatory [Park et al., 2005; Kim et al., 2007; Sung et al., 2008], anti-nociceptive [Park et al., 2005], and anti-tumorigenic [Kim et al., 2006; Nakajima et al., 2009a] activities. 3,4-Dihydroxybenzalacetone (DBL) is a small catechol-containing compound isolated and purified from 80% of MeOH extract of the Chaga fruiting body, and expected to be the major antioxidant ingredient in Chaga [Nakajima et al., 2007]. DBL has been reported to have antioxidative [Nakajima et al., 2007, 2009a], anti-inflammatory [Sung et al., 2008], and anti-tumorigenic properties [Nakajima et al., 2009a], with relatively low toxicity to normal cells. The hydrogen peroxide-induced cell death in rat adrenal pheochromocytoma PC12 cells was more effectively prevented in cells treated with DBL than in cells treated with caffeic acid (CA), which is another Chaga-derived catechol-containing small compound [Nakajima et al., 2009b].

In this study, we investigated the neuroprotective activity of DBL against 6-OHDA neurotoxicity using neuroblastoma SH-SY5Y cells, which express dopaminergic neuron-associated genes such as DAT and Vesicular Monoamine Transporter 2 (VMAT2), and are commonly used to make a cell culture model of PD [Takahashi et al., 1994; Pan et al., 2005]. Unlike a typical anti-oxidant, N-acetyl cysteine (NAC), DBL did not directly quench 6-OHDA-induced ROS in cell-free system. However, it effectively induced intracellular defense systems against oxidative stress, especially in the nuclear factor erythroid 2-related factor 2 (Nrf2)/glutathione pathway, and improved survival of SH-SY5Y cells against 6-OHDA. Activation of phosphatidylinositol 3-kinase (PI3K)/Akt pathway was most likely involved in the upstream of Nrf2.

MATERIALS AND METHODS

MATERIALS

DBL was synthesized at Niigata University of Pharmacy and Applied Life Sciences as described in the Supplementary Information. The purity of DBL was more than 99.9% when evaluated by HPLC analysis. CA and buthionine sulfoximine (BSO), an inhibitor of γ-glutamyl cysteine ligase (γ-GCL), were purchased from Wako Pure Chemical Industries. (Osaka, Japan). 6-OHDA and NAC were purchased from Sigma (St Louis, MO). Hydrogen peroxide (H_2O_2) and MG132, a proteasome inhibitor, were purchased from Nacalai Tesque (Kyoto, Japan). Sn-mesoporphyrin IX (Sn-MP), a heme oxygenase-1 (HO-1) inhibitor, and dicoumarol, an NAD(P)H:quinone oxidoreductase 1 (NQO1) inhibitor, were purchased from Frontier Scientific, Inc. (Logan, UT) and Tokyo Chemical Industry Co. (Tokyo, Japan), respectively. SB202190, a p38 Mitogen-activated protein kinase (MAPK) inhibitor, LY294002, a Phosphatidylinositol 3-kinase inhibitor, SP600125, a Jun amino-terminal kinase (JNK) inhibitor, and PD98059, an Extracellular signal-regulated kinase (ERK) inhibitor, were purchased from Sigma. Akt inhibitor IV was purchased from Millipore (Billerica, MA).

CELL CULTURE

A human neuroblastoma cell line, SH-SY5Y, was maintained in DMEM containing 15% FBS and 50 μg/ml of penicillin and 100 μg/ml of streptomycin. These cells were kept at 37°C in humidified 5% CO_2/95% air.

STRESS CONDITION

SH-SY5Y cells were cultured in 24-well plates (MTT assay and LDH assay) or in 6-well plates (qRT-PCR, Western blot, and total glutathione measurement) up to 60–70% confluence. Cells were pre-treated with DBL, CA, or other compounds for the indicated times (1–8 h), and after washing with PBS, they were exposed to 6-OHDA (30 μM) or maintained in the medium alone for the indicated times (6–24 h). In some experiments, DBL or CA was incubated in the culture medium (cell-free condition) for 1 h in the presence or absence of 6-OHDA, and ROS production was monitored by FOX assay as described below.

CELL VIABILITY AND CELL DEATH ASSAYS

Cell viability and cell death were measured by MTT assay (Nacalai Tesque) and LDH cell toxicity assay (Kyokuto Pharmaceutical Industrial, Tokyo, Japan), respectively. In the latter experiment, the LDH value in each condition was subtracted from the LDH value in the medium alone.

MEASUREMENT OF ROS

The level of the intracellular ROS after 6-OHDA treatment was measured using a fluorescent probe, 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA; Invitrogen) as described before [Takano et al., 2007]. In brief, SH-SY5Y cells were loaded with 5 μM DCFH-DA for 20 min, and after washing with PBS, the fluorescence was measured in a microscope (Nikon TS100-F ECLIPSE) equipped with a CCD camera (Hamamatsu Photonics, Shizuoka, Japan). Quantification of the fluorescent intensity was performed using Image J (version 1.42, Wayne Rasband, National Institutes of Health), and the results were shown as percentages of the control intensity. The level of ROS in vitro was measured using FOX assay as previously described [Wagner et al., 2011]. A standard curve was prepared with H_2O_2 (0, 2.2, 8.8, 2.2, and 88 μM).

CELL LYSIS AND WESTERN BLOTTING

Cells were lysed in RIPA buffer containing 10 mM Tris, 1 mM EDTA, 150 mM NaCl, 1% NP-40, 0.1% SDS, 0.2% sodium deoxycholate, and

protease inhibitors. Samples were then subjected to Western blotting with the following antibodies: HO-1 (Abcam, Cambridge, UK), cystine/glutamate antiporter (xCT) (Thermo Scientific, Rockford, IL), glutamate-cysteine ligase, modifier subunit (GCLM) (Proteintech Group, Chicago, IL), p-ERK, ERK, p-JNK, JNK, p-Akt, Akt, p38 (Cell Signaling Technology, Beverly, MA), p-p38 (Santa Cruz Biotechnology, Santa Cruz, CA), and β-actin (Sigma). For analyzing translocation of Nrf2 protein, cytosol and nuclear fractions were isolated as previously described [Schreiber et al., 1989], and protein extracts were subjected to Western blotting with anti-Nrf2 antibody (Santa Cruz Biotechnology). Sites of primary antibody binding were visualized using the ECL system (GE Healthcare Bio-Sciences Corp., Piscataway, NJ). Quantification of the intensity of each band was performed using Image J, and the results were shown as the ratio to the intensity of β-actin.

QUANTITATIVE REAL TIME RT-PCR (QRT-PCR)

Total RNA was extracted from SH-SY5Y cells using RNAzol®RT (Invitrogen). RT reactions containing 1 μg of total RNA were performed using PrimeScript (Takara, Shiga, Japan). The individual cDNA was amplified with THUNDERBIRD™ SYBR qPCR® Mix (Toyobo Co, Ltd, Osaka, Japan) by using the following specific primers: HO-1, NQO-1, xCT, GCLM, and β-actin. The comparative Ct method was used for data analyses with MxPro 4.10 (Agilent Technologies, Santa Clara, CA). Values for each gene were normalized to β-actin expression levels.

MEASUREMENT OF TOTAL GLUTATHIONE

Total glutathione (GSH/GSSG) contents were measured using Total Glutathione Quantification Kit (Dojindo, Kumamoto, Japan). Briefly, SH-SY5Y cells were pre-treated with DBL or CA for 4 h, and further incubated with 6-OHDA or medium alone for 16 h. In some cases, cells were also treated with BSO in the presence or absence of 6-OHDA for 16 h after pretreatment with DBL for 4 h. Cells were then harvested, and lysed in 0.2 ml of PBS using a sonicator (Braoson, Danbury, CT). The samples were deproteinized with 20 μl of 5% 5-sulfosalicylic acid (Sigma), and total glutathione was measured by following the manufacturer's instructions.

LUCIFERASE ASSAY

A 15-kb HO-1 promoter DNA linked to firefly luciferase reporter gene (pOH15luc) was kindly provided by Dr. Jawed Alam (Ochsner Clinic Foundation, New Orleans, LA). A constitutively active *Renilla* luciferase (pRL-SV40) was purchased from Promega (Madison, WI). Transient transfection was performed in SH-SY5Y cells using lipofectAMINE 2000 reagent (Invitrogen-Life Technologies, Carlsbad, CA). Two days after transfection, cells were treated with DBL for 4 h, followed by incubation in the medium in the presence or absence of 6-OHDA for 6 h. The activities of firefly and *Renilla* luciferase were measured using a dual luciferase assay kit (Promega). The ratio of two luciferase activities was taken as the promoter activity.

STATISTICAL ANALYSIS

Statistical analyses were performed using a Bonferroni/Dunn test following a one-way ANOVA. Differences were considered statistically significant when *P*-values were less than 0.05.

RESULTS

PROTECTION OF SH-SY5Y CELLS BY DBL AGAINST 6-OHDA NEUROTOXICITY

To analyze the neuroprotective effect of DBL, SH-SY5Y cells were pre-treated with DBL or CA (Fig. 1A I) for 4 h, and after washing with PBS, cells were treated with 30 μM 6-OHDA (Fig. 1A II) for 24 h. Cell viability and cell death were measured using MTT assay (Fig. 1B) and LDH assay (Fig. 1D), respectively. 6-OHDA decreased cell viability to 40–50% of the control cells (Fig. 1B). Pretreatment of the cells with DBL, but not with CA, significantly improved cell viability against the toxicities induced by 6-OHDA in a dose-dependent manner (Fig. 1B). The protective role of DBL was observed following as short as 1 h of pretreatment, and it increased after 4 and 8 h of pretreatment (Fig. 1C). Consistent with these results, 6-OHDA-induced cell death was attenuated by pretreatment with DBL, but not with CA (Fig. 1D). These results suggest that the hydrophobicity of the side chain in DBL may play an important role in its cell-protecting activity.

EFFECT OF DBL ON THE ROS GENERATION

To analyze whether the neuroprotective activity of DBL is associated with its antioxidative property, a fluorescent probe DCFH-DA was employed to measure the level of ROS in SH-SY5Y cells (Fig. 2A). 6-OHDA-induced ROS production (approximately 36-fold increase of DCF) was dose-dependently reduced by pretreatment of the cells with DBL (approximately 4-fold increase of DCF) but not by CA (approximately 30-fold increase of DCF). However, the effect was slightly weaker than that of NAC (approximately 1.5-fold increase of DCF), the latter was a well-known antioxidant that has both glutathione- and ROS-quenching effects [Arakawa and Ito, 2007]. In the cell-free system, 6-OHDA also induced ROS production (approximately 40 μM of hydrogen peroxide), probably through its auto-oxidation, as described previously [Hanrott et al., 2006]. Although NAC reduced 6-OHDA-mediated ROS production in this system, neither DBL nor CA showed similar effect (Fig. 2B II and III). Instead, both of DBL and CA mildly enhanced ROS production in the absence of 6-OHDA, while the effect was much lower than that of 6-OHDA (Fig. 2B II). These results suggest that the direct quenching effect of DBL was not strong enough to scavenge ROS produced in the reaction system, but DBL, as a mild pro-oxidant, may activate intracellular defense systems against oxidative stress, and indirectly reduce the ROS in the cells.

EFFECT OF DBL ON THE OXIDATIVE STRESS RESPONSE

Many phenolics activate intracellular defense systems against oxidative stress, especially in the Nrf2 pathway. Therefore, the effect of DBL and CA on the oxidative stress response was estimated by analyzing expressions of antioxidative genes and Phase II detoxification genes both at mRNA and protein levels. qRT-PCR analysis revealed that DBL upregulated the expression of Nrf2-downstream genes such as HO-1, xCT, NQO1, GCLC, and GCLM (Fig. 3A), but not other antioxidant genes such as Mn-SOD (data not shown) at the mRNA level, both in the presence and absence of 6-OHDA. Consistently, the expression of HO-1, xCT, and GCLM protein was significantly enhanced by DBL, both in the presence and absence of 6-OHDA (Fig. 3B). In contrast, CA had much less effect on the

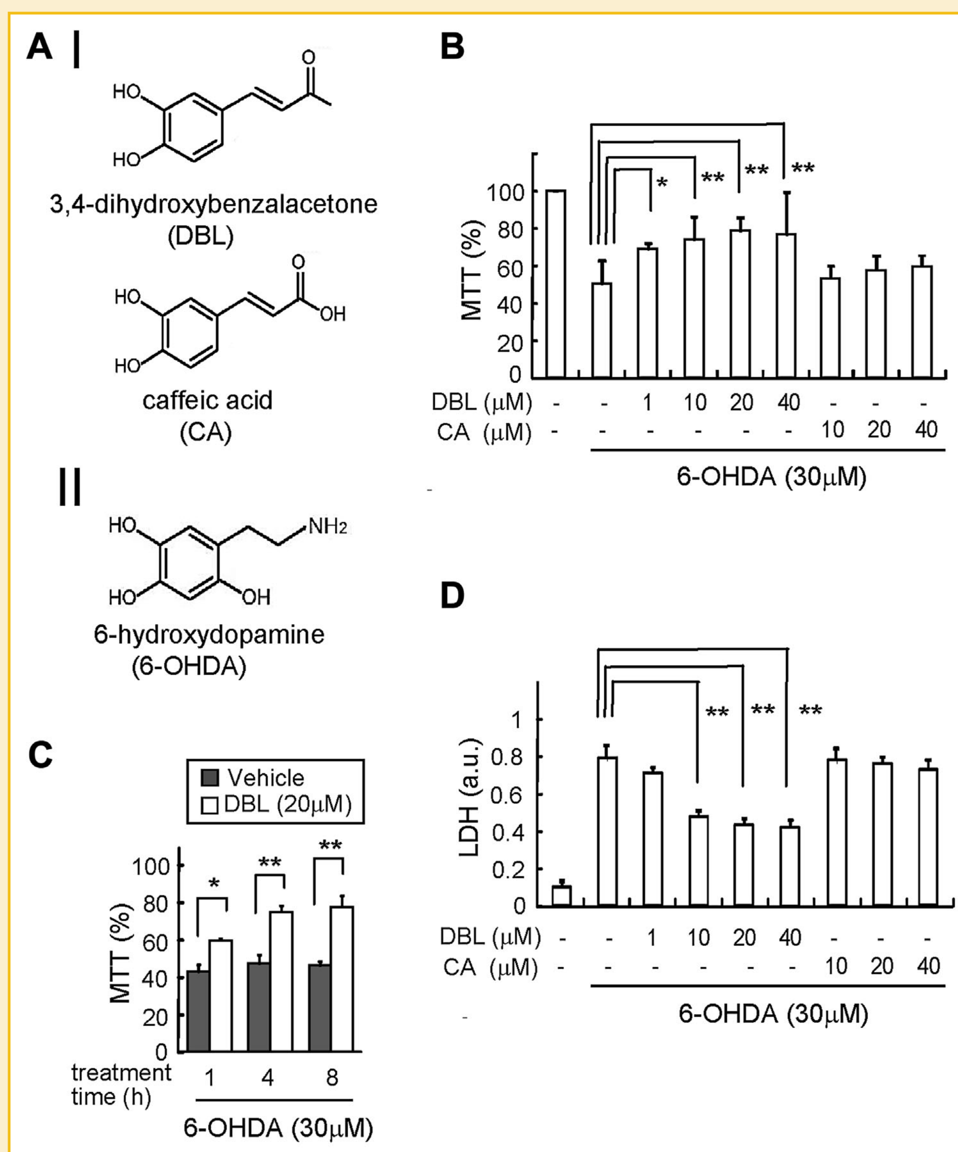

Fig. 1. Protective effect of DBL against 6-OHDA toxicity in SH-SY5Y cells. (A) Structure of DBL, CA (I) and 6-OHDA (II). (B and C) Improved viability in DBL-pretreated cells after 6-OHDA treatment. SH-SY5Y cells were pretreated with DBL or CA (0–40 μM) for 4 h (B), or DBL (20 μM) for the indicated times (C), and treated with 6-OHDA (30 μM) for 24 h. Cell viability was measured using MTT assay. Values shown are the mean ± SD in four separate experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$, significantly different between two conditions. (D) Reduced cell death in DBL-pretreated cells after 6-OHDA treatment. SH-SY5Y cells were pretreated with DBL or CA (0–40 μM) for 4 h, and treated with 6-OHDA (30 μM) for 24 h. Cell death was measured using LDH assay. Values shown are the mean ± SD in four separate experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$, significantly different between two conditions.

expression of the above genes, when compared to DBL (Fig. 3A and B). The total glutathione content was also significantly increased by DBL, but not by CA, both in the presence and absence of 6-OHDA (Fig. 3C).

To analyze the effect of DBL on Nrf2 activation, the translocation of Nrf2 from the cytosol to the nucleus was assessed by Western blotting. DBL dose-dependently increased Nrf2 translocation in the presence and absence of 6-OHDA (Fig. 3D). Consistent with this, a reporter analysis using HO-1 promoter, which includes antioxidant response element (ARE), revealed that DBL indeed enhanced HO-1 reporter activity in a dose-dependent manner, both in the presence and absence of 6-OHDA (Fig. 3E).

ROLE OF GLUTATHIONE SYNTHESIS IN DBL'S NEUROPROTECTIVE EFFECT

To identify the molecule(s) critical for the neuroprotective role of DBL, cells were treated with several inhibitors, including BSO, a γ-GCL inhibitor, Sn-MP, a HO-1 inhibitor, and Dicoumarol, a NQO1 inhibitor, in the presence or absence of 6-OHDA after pretreatment with DBL. Treatment of the cells with BSO (200 μM) reduced intracellular glutathione content to approximately 20% of control (BSO free) condition, but did not cause cell death in the absence of 6-OHDA (data not shown). In contrast, BSO reduced cell viability, and, more importantly, completely abolished the protective effect of DBL

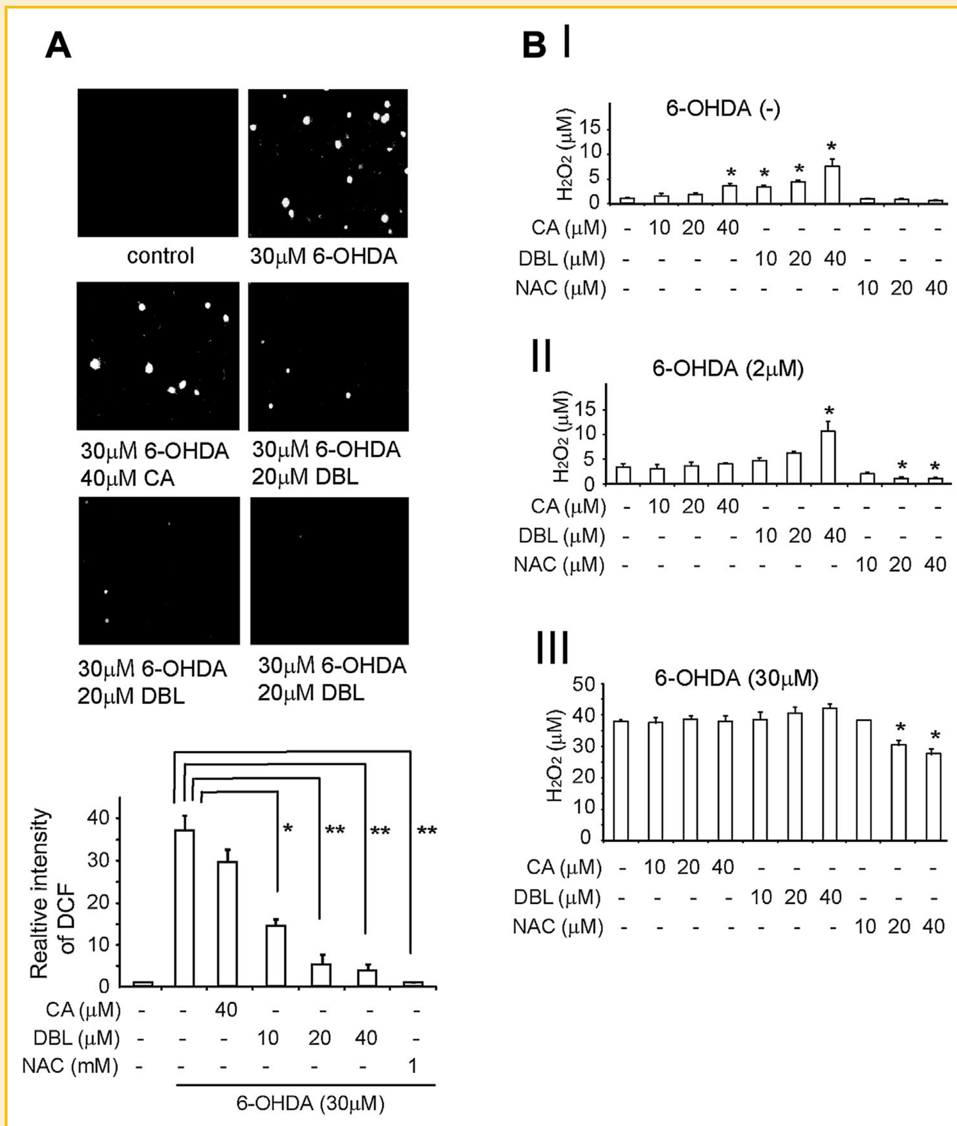

Fig. 2. Effect of DBL and CA on 6-OHDA-induced ROS generation. (A) Effect of DBL and CA on ROS generation in SH-SY5Y cells. Cells were pretreated with DBL (0, 20, and 40 μM), CA (40 μM), or NAC (1 mM) for 4 h, and treated with 6-OHDA (30 μM) for 6 h. The level of ROS was measured using fluorescent dye DCFH-DA, and quantified using Image J. Values shown are the mean $\pm$ SD in four separate experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$, significantly different between two conditions. (B) Effect of DBL and CA on ROS generation in vitro. DBL (0–40 μM), CA (0–40 μM), or NAC (0–40 μM) were incubated in the medium (15% FBS DMEM) in the presence or absence of 6-OHDA for 1 h. Concentration of hydrogen peroxide (H_2O_2) in the medium was then measured using FOX assay. Values shown are the mean $\pm$ SD in four separate experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$, significantly different from values obtained under incubations in the medium alone.

in the presence of 6-OHDA (Fig. 4A). In contrast to BSO, Sn-MP (40 μM) or Dicoumarol (20 μM) did not affect the protective role of DBL against 6-OHDA toxicity (Fig. 4B and C).

ROLE OF PROTEIN KINASES ON GLUTATHIONE SYNTHESIS AND NEUROPROTECTION BY DBL

To investigate the upstream event in the Nrf2 pathway, the activation status of several protein kinases was analyzed by Western blotting. Treatment of cells with DBL, but not with CA, induced phosphorylation of Akt, ERK, and p38 in 1 h (Fig. 5A). In contrast, the phosphorylation of JNK was not induced in the same condition (Fig. 5A). To further elucidate the effect of protein kinase activation on neuroprotection, glutathione synthesis, and Nrf2 activation by DBL, cells were treated with DBL, together with relatively low dose (1–2 μM) of protein kinase inhibitors such as LY294002, a PI3K inhibitor, Akt inhibitor IV, PD98059, an ERK inhibitor, SB202190, a p38 inhibitor, or SP600125, a JNK inhibitor, for 4 h, followed by incubation in the medium, in the presence or absence of 6-OHDA. The effect of DBL on cell viability (Fig. 5B), glutathione synthesis (Fig. 5C) and Nrf2 activation (Fig. 5D) was all abolished by LY294002 or Akt inhibitor IV, but not by other protein kinase inhibitors, suggesting involvement of

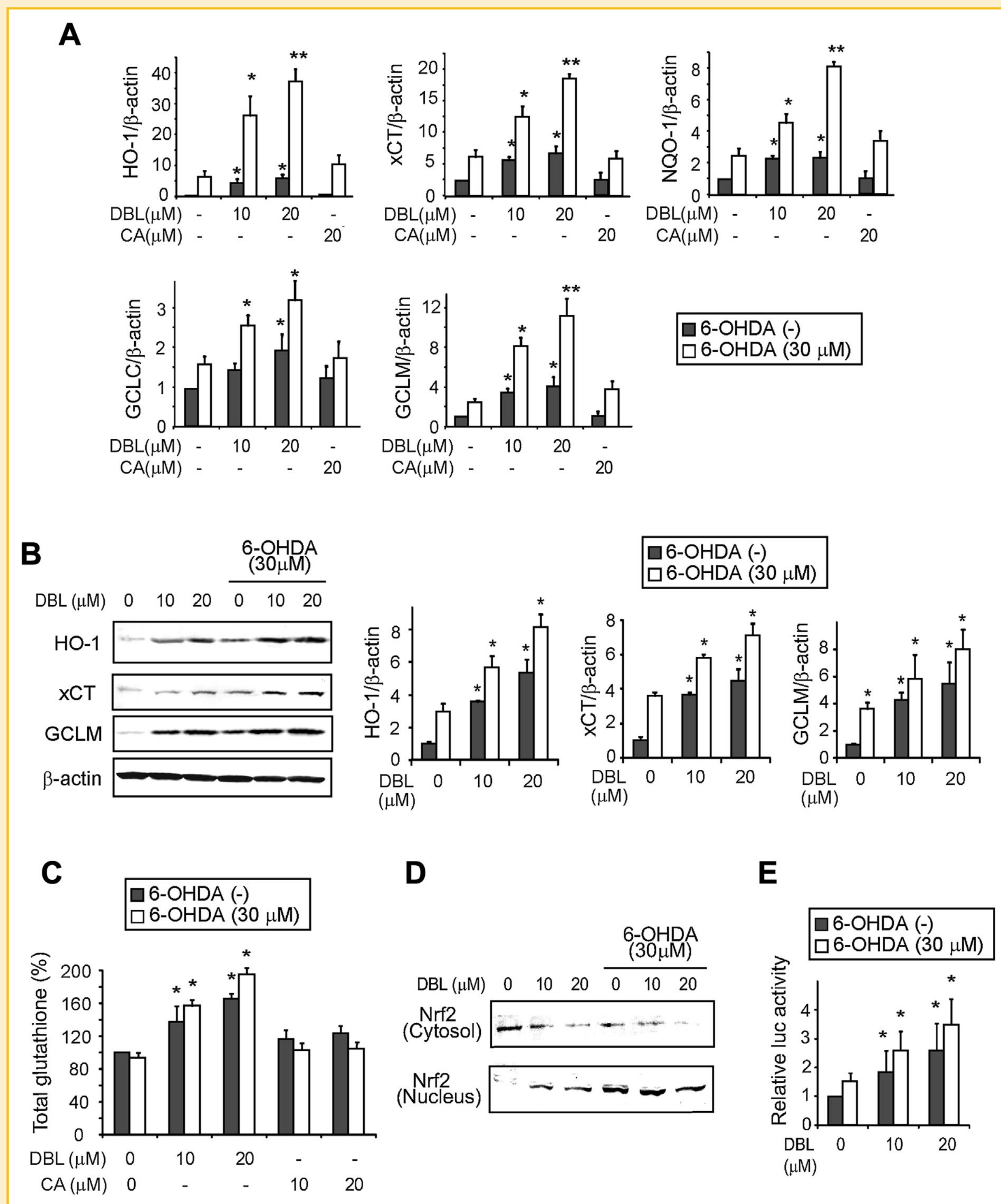

Fig. 3. Effects of DBL and CA on the oxidative stress response. (A) Expression of antioxidant and Phase II detoxification transcripts. Cells were treated with DBL or CA for 4 h, and then incubated in the medium in the presence or absence of 6-OHDA (30 μM) for 6 h. Total RNA was extracted, and qRT-PCR was performed with specific primers for the indicated genes. Values shown are the mean ± SD in four separate experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$, significantly different from values in the conditions without DBL or CA treatment. (B) Expression of antioxidant and Phase II detoxification proteins. Cells were treated with DBL or CA for 4 h, and then incubated in the medium in the presence or absence of 6-OHDA (30 μM) for 10 h. Protein extracts were subjected to Western blotting with indicated antibodies. The intensity of each band was quantified using Image J. Values shown are the mean ± SD in four separate experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$, significantly different from values in the conditions without DBL or CA treatment. (C) Total glutathione levels in the cells. Cells were treated with DBL or CA for 4 h, and then incubated in the medium in the presence or absence of 6-OHDA (30 μM) for 16 h. The amount of total glutathione was measured as described in the text. Values shown are the mean ± SD in four separate experiments. $^{*}P < 0.05$, significantly different from values in the conditions without DBL or CA treatment. (D and E) Translocation of Nrf2 into the nucleus (D) and activation of ARE-containing HO-1 promoter (E). Cells were treated with DBL for 4 h, and then incubated in the medium in the presence or absence of 6-OHDA (30 μM) for 6 h. Both cytosolic and nuclear fractions were subjected to Western blotting with anti-Nrf2 antibody (D). Luciferase assay was also performed as described in the text (E). In the latter experiment, values shown are the mean ± SD in four separate experiments. $^{*}P < 0.05$, significantly different from values in the conditions without DBL treatment.

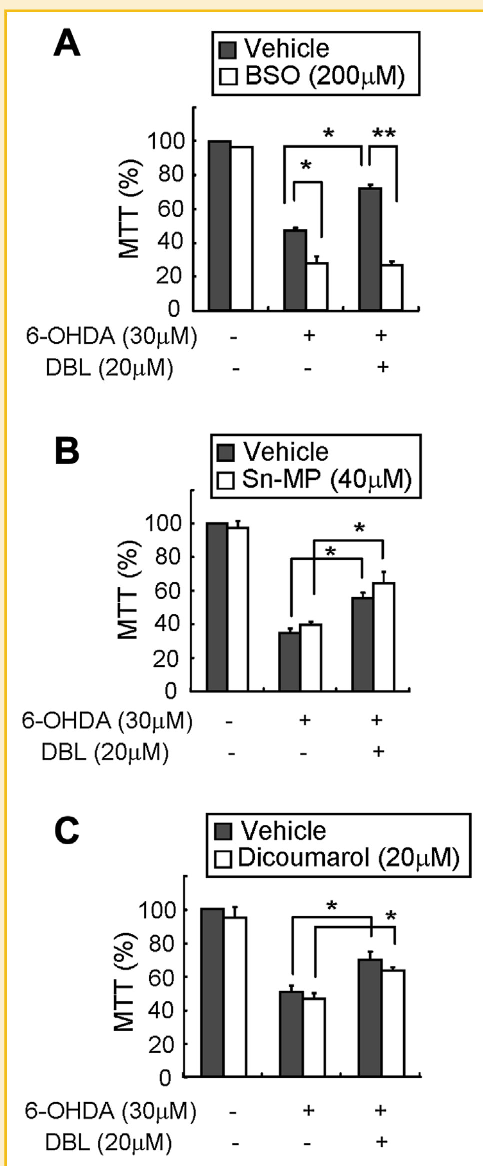

Fig. 4. Effect of antioxidants and Phase II detoxification enzyme on neuroprotection by DBL. Cells were pretreated with DBL (20 μM) or with medium alone for 4 h, and then treated with several inhibitors, including BSO (A), Sn-MP (B), and dicoumarol (C) in the presence or absence of 6-OHDA for 24 h. Cell viability was measured using MTT assay. Values shown are the mean ± SD in four separate experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$, significantly different between two conditions.

PI3K/Akt activation in Nrf2 activation, glutathione synthesis, and neuroprotection by DBL.

DISCUSSION

In this study, we investigated the neuroprotective effect of DBL against PD-related neurotoxin 6-OHDA in SH-SY5Y cells. Pretreatment of the cells with DBL, but not with CA, dose-dependently improved the viability of cells treated with 6-OHDA. The mechanism may include enhancement of the intracellular defense systems against oxidative stress. DBL attenuated 6-OHDA-induced ROS production in SH-SY5Y cells (Fig. 2A), but not in the cell-free system (Fig. 2B). Instead, DBL activated Nrf2 (Fig. 3D and E), and upregulated the expression of Nrf2-dependent antioxidative genes (Fig. 3A and B) and increased glutathione synthesis (Fig. 3C). Treatment of the cells with BSO abolished the protective effect of DBL against 6-OHDA (Fig. 4A). Furthermore, DBL activated Akt (Fig. 5A), and an inhibitor of PI3K or Akt diminished DBL-induced glutathione synthesis and protection against 6-OHDA (Fig. 5B–D).

The medicinal properties of Chaga have been extensively studied in Russia, using various experimental animals, as well as humans [Shashkina et al., 2006]. It is now accepted that the toxicity of Chaga is quite low, and daily intake of Chaga is beneficial for health promotion and for the long-term treatment of patients with gastrointestinal ulcers and uterine cancer [Shashkina et al., 2006]. Interestingly, DBL exhibited greater toxicity toward cancer cells and markedly less toxicity toward normal cells [Nakajima et al., 2009a]. Among small phenolic compounds extracted from Chaga, DBL is found only in fungi, and therefore expected to be the major antioxidant ingredient in Chaga [Nakajima et al., 2007]. DBL suppressed H_2O_2-induced oxidative stress and p38-mitogen activated protein kinase (MAPK) in rat adrenal pheochromocytoma PC12 cells [Nakajima et al., 2009b]. The discrepancy regarding the mechanism of the cell protection by DBL between the latter report and the present study may lie in the difference in the experimental conditions in addition to different cell types. In the previous study, PC12 cells was exposed to relatively high dose of H_2O_2 (0.3 mM), while in the present study, SH-SY5Y cells were treated with a lower dose of 6-OHDA (30 µM). Furthermore, the treated period of the cells with DBL was 1 h in the previous study, while 4 h in most cases of the present study, based on the observation that 4- to 8-h treatment showed a higher protective effect than did 1 h of treatment (Fig. 1C).

6-OHDA, DBL, and CA are all catechol-containing compounds (Fig. 1A I and II), but their biological functions were quite different due to the structure of side chains. 6-OHDA has more oxidizable phenols than DBL or CA, and undergoes non-enzymatic oxidization (auto-oxidation), which generates *p*-quinones, hydrogen peroxide, superoxide anions, and hydroxyl radicals in the extracellular milieu [Blum et al., 2001; Hanrott et al., 2006]. In the current study, 6-OHDA (30 µM) generated approximately 40 µM of hydrogen peroxide within 1 h in the cell-free system (Fig. 2B III), and caused cell death in SH-SY5Y cells within 24 h (Fig. 1B–D). In contrast, DBL (10–40 µM) generated, if any, less than 5 µM of hydrogen peroxide in the same condition (Fig. 2B I), while effectively activated Nrf2 and enhanced the expression of downstream antioxidative genes in SH-SY5Y cells (Fig. 3A–E). It is likely that the PI3K/Akt pathway plays an important role for both Nrf2 activation and cell protection against 6-OHDA by DBL (Fig. 5A–D). Presently, however, we do not rule out the possibility that Michael acceptor (olefins or acetylenes conjugated with electron-withdrawing carbonyl groups) in the propene side chain and/or catechol-derived oxidizable phenols of DBL directly inhibit the interaction of Kelch-like ECH-associated protein 1 (Keap) and Nrf2, leading to the translocation of Nrf2 to the nucleus [Magesh et al., 2012].

Although CA has the same structure as DBL (bearing catechol enone moiety in the molecule), except for the end of the propene side

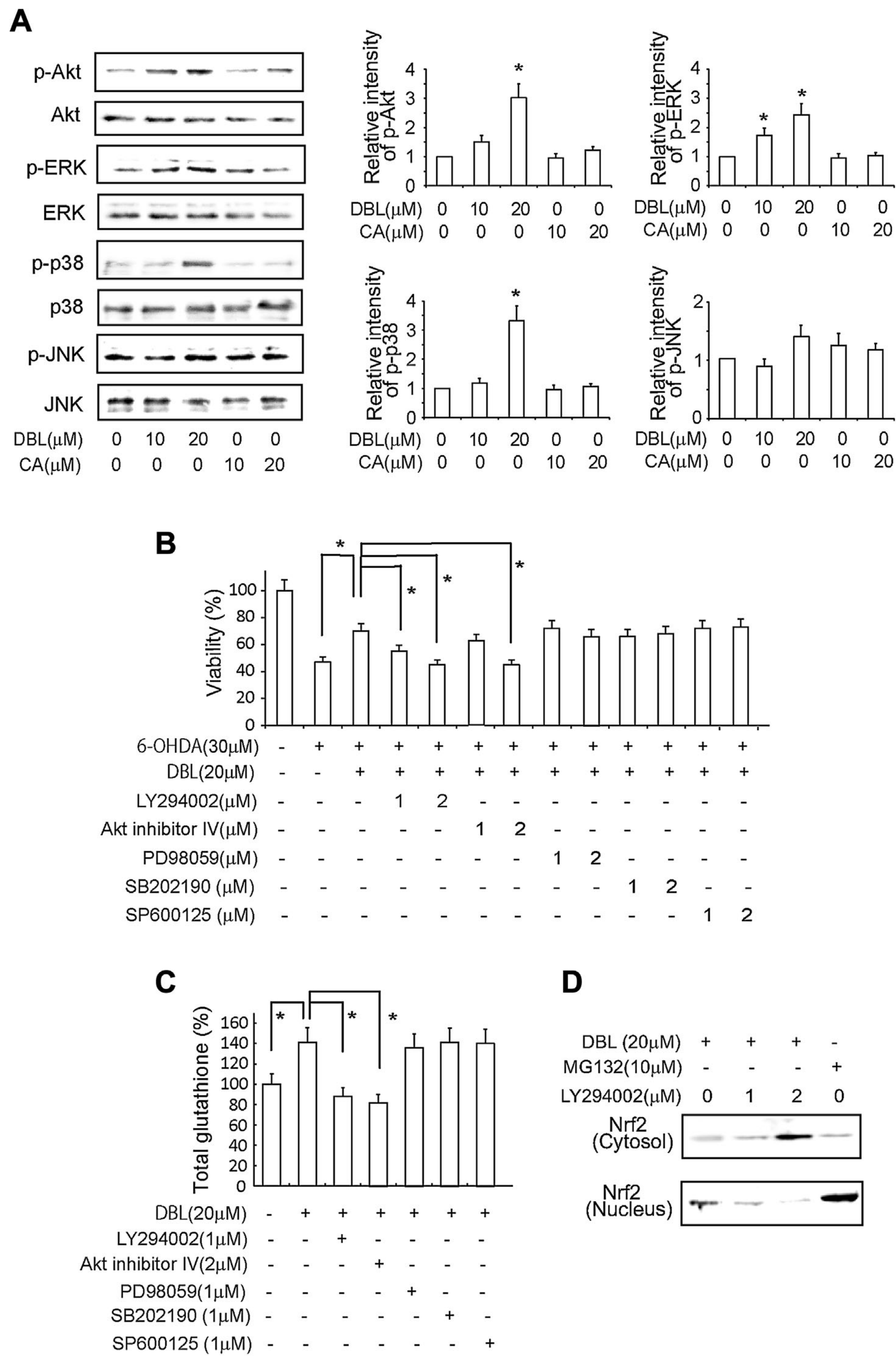

Fig. 5. Activation of the protein kinases by DBL. (A) Phosphorylation of stress-associated kinases after DBL treatment. Cells were treated with DBL, CA or cultured in the medium alone for 1 h, and protein extracts were subjected to Western blotting using the indicated antibodies. Relative intensity was measured using Image J as described in the text. (B–D) Effects of kinase inhibitors on BDL-mediated cell protection (B), glutathione synthesis (C), and Nrf2 activation (D). Cells were treated with the indicated kinase inhibitors in the presence of DBL for 4 h, and incubated in the medium in the presence or absence of 6-OHDA for 24 h (B), 16 h (C), and 6 h (D). The cell viability (B), the amount of total glutathione (B), and the translocation of Nrf2 into the nucleus (D) were measured. MG132 was used as a positive control. Values shown are the mean ± SD in four separate experiments. $^{*}P < 0.05$, significantly different between two conditions.

chain (Fig. 1A I), it failed to protect SH-SY5Y cells against 6-OHDA in our model (Fig. 1B and D). Furthermore, CA could not induce antioxidative genes to the levels that DBL did in SH-SY5Y cells (Fig. 3A). These results suggest that the hydrophobicity of the propene side chain in DBL may play an important role for its membrane permeability or its signal-activating property in the cells. Consistent with this hypothesis, caffeic acid phenethyl ester (CAPE), another catechol-containing compound with a hydrophobic side chain [Celli et al., 2007], was reported to activate the Nrf2 signaling and protect neurons against oxidative stress [Scapagnini et al., 2011]. There are also reports regarding the anti-tumorigenic, anti-inflammatory, and immunomodulatory properties of CAPE [Watanabe et al., 2011]. Further studies are required to understand how subtle changes in the side chain of DBL affect its functions.

The current study also emphasizes the important role of glutathione in the protective property of DBL against 6-OHDA toxicity. Glutathione is the most abundant antioxidant in the brain, which serves to detoxify H_2O_2 to water and oxygen, and keeps the thiol groups of proteins in a reduced state [Mytilineou et al., 2002]. Numerous studies have demonstrated decreased levels of glutathione in pathological conditions, including brain ischemia and neurodegenerative diseases such as PD. Postmortem studies have revealed that glutathione levels in the substantia nigra (SN) are decreased with age, and dramatically reduced in the brains of PD patients, in concert with the death of dopaminergic neurons in the SN [Perry and Yong, 1986; Riederer et al., 1989]. In experimental animal studies, acute depletion of glutathione causes extensive carbonylation of brain proteins [Bizzozero et al., 2006] and morphological changes of dopaminergic neurons in the nigrostriatal pathway, which mimic alterations observed in the brains of aged animals [Andersen et al., 1996]. Our next goal is, therefore, to assess whether DBL can activate the Nrf2-glutathione pathway and protect dopaminergic neurons in vivo. We also plan to study the anti-inflammatory role of DBL in neurodegenerative conditions.

In conclusion, we have demonstrated that DBL enhances intracellular defense systems against oxidative stress, especially the Nrf2-glutathione pathway through PI3K/Akt, and improves the survival of SH-SY5Y cells treated with 6-OHDA.

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