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Research Article

Decursin Isolated from *Angelica gigas* Nakai Rescues PC12 Cells from Amyloid β-Protein-Induced Neurotoxicity through Nrf2-Mediated Upregulation of Heme Oxygenase-1: Potential Roles of MAPK

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Decursin (D), purified from *Angelica gigas* Nakai, has been proven to exert neuroprotective property. Previous study revealed that D reduced Aβ_{25-35}-induced cytotoxicity in PC12 cells. Our study explored the underlying mechanisms by which D mediates its therapeutic effects *in vitro*. Pretreatment of cells with D diminished intracellular generation of ROS in response to Aβ_{25-35}. Western blot revealed that D significantly increased the expression and activity of HO-1, which was correlated with its protection against Aβ_{25-35}-induced injury. Addition of ZnPP, an HO-1 competitive inhibitor, significantly attenuated its protective effect in Aβ_{25-35}-treated cells, indicating the vital role of HO-1 resistance to oxidative injury. Moreover, D induced Nrf2 nuclear translocation, the upstream of HO-1 expression. While investigating the signaling pathways responsible for HO-1 induction, D activated ERK and dephosphorylated p38 in PC12 cells. Addition of U0126, a selective inhibitor of ERK, blocked D-induced Nrf2 activation and HO-1 induction and meanwhile reversed the protection of D against Aβ_{25-35}-induced cell death. These findings suggest D augments cellular antioxidant defense capacity through both intrinsic free radical scavenging activity and activation of MAPK signal pathways that leads to Nrf2 activation, and subsequently HO-1 induction, thereby protecting the PC12 cells from Aβ_{25-35}-induced oxidative cytotoxicity.

1. Introduction

Alzheimer's disease (AD) is the most common form of senile dementia, affecting millions of people worldwide. It is an age-related neurodegenerative disease pathologically characterized by deposition of senile plaques, intracellular neurofibrillary tangles (NFT), and loss of neurons in the brain. Amyloid β-peptide (Aβ), a 39- to 43-amino acid peptide fragment derived from an amyloid precursor protein *via* a sequential cleavage by β- and γ-secretases, is the major component of senile plaques and is considered to be tightly related to the development and progress of AD. Extensive evidence indicates that the brains of individuals with AD are characterized by exaggerated oxidative stress [1–8], and the overproduction of Aβ leads to Aβ-associated free radical production and cell death [9–13]. Not only does Aβ increase oxidative stress, but its generation is also increased as a result of oxidative stress, which in turn causes more oxidative damage. Given the important role of oxidative stress in AD, therapeutic strategies which are directed at early interventions targeted at oxidative stress may be effective in delaying AD development and slowing its progression. Indeed, increased antioxidant activity confers protection and has been reported to lower the risk of AD [14]. Thus, an approach which simultaneously enhances various intracellular oxidative defense capacities may be more effective in combating neurodegeneration.

Among the various cytoprotective enzymes, heme oxygenase (HO) has received considerable attention, which consists of three isoforms: HO-1, HO-2, and HO-3. Although HO-2

and HO-3 are constitutively expressed, HO-1 is inducible in many cell types, such as neuronal cells [15, 16]. HO-1 is one of the major antioxidant/cytoprotective enzymes that are readily induced in response to oxidative stress. HO-1 catalyzes the rate-limiting step in the heme degradation process, releasing iron, carbon monoxide (CO), and biliverdin. The antioxidant potential of HO-1-generated metabolic products highlights the HO-1 pathway as a therapeutic target for pharmacological intervention of various diseases including neurological disorders [17–19]. The induction of HO-1 resulted in a relatively higher resistance to glutamate- and H_2O_2-mediated oxidative damage and MPTP- or Aβ-induced neurotoxicity [20–23]. Transcriptional regulation of the *ho-1* gene is linked to the transcription factor NF-E2-related factor (Nrf2), which plays a crucial role in cellular defense. Nrf2 is a basic leucine zipper transcription factor that resides in the cytoplasm bound to its inhibitor protein, Keap1, and translocated to the nucleus after stimulation. It then binds to the antioxidant response element (ARE) sequences in the promoter regions of cluster of antioxidant/detoxifying genes, such as *ho-1* [24–26]. Activation of Nrf2 pathway has been demonstrated to be involved in the protection of the nerve cells against oxidative damage *in vivo* and *in vitro* [27–29]. Neurons lacking Nrf2 are highly sensitive to oxidative stress but can be rescued by transfection with a functional Nrf2 construct [30]. In addition, activation of the Nrf2/ARE pathway in astrocytes by tert-butylhydroquinone (tBHQ), an Nrf2 activity inducer, is able to protect neurons from subsequent oxidative stress [31].

To date, multiple signaling kinases related to cell survival/proliferation have been reported to regulate the nuclear translocation of Nrf2, including mitogen-activated protein kinases (MAPKs), phosphatidylinositol 3-kinase (PI3K), and protein kinase C (PKC) [32–34]. MAPK is one of the most common signaling pathways that serve to coordinate the cellular response to a variety of extracellular stimuli. These are well characterized in mammals and include *c*-Jun N-terminal kinase (JNK), p38 MAP kinase (p38), and mitogenic signaling, extracellular signal-regulated kinase 1/2 (ERK). Several members of MAPK family are implicated in neurodegenerative processes [35] and its activation also modulates several gene and protein expressions, such as that of HO-1 [36, 37].

Thus, in light of the cytoprotective role of HO-1, the specific activation of Nrf2 and upregulation of HO-1 gene expression by pharmacological modulation may represent a novel target form therapeutic intervention of AD. *Angelica gigas* Nakai (Umbelliferae) root is used in traditional oriental herbal medicine to treat female afflictions and is regarded by herbalists as female ginseng for its hemopoietic and health-promoting activities [38]. Decursin (D) is a pyranocoumarin which is the major active ingredient present in *Angelica gigas* Nakai. Reported *in vivo* and *in vitro* studies indicated that D possesses anticancer [39–43], antibacterial [44], antiplatelet aggregation [45], antinematodal activities [46], and antioxidant activities [47] properties. Growing bodies of evidence have supported the fact that D exhibits potent neuroprotective activity against glutamate-induced neurotoxicity in primary cultures of rat cortical cells [48] and greatly improves scopolamine-induced amnesia in mice [49, 50]. Our previously study indicated that D increases cellular resistance to Aβ-induced oxidative injury in the rat pheochromocytoma (PC12) cells, presumably through not only the induction of Nrf2 and related antioxidant enzymes, but also the antiaggregation of Aβ [51]. However, the upstream signaling and the detailed molecular mechanisms by which D exerts its neuroprotective effects *in vivo* remain largely unresolved. To gain a further insight into the biological roles of D, we attempt, in this study, to elucidate the correlation between its neuroprotection effect and HO-1 production. We designed an experiment to investigate whether the D-induced HO-1 expression is associated with the activation of MAPKs/Nrf2 in PC12 cells following treatment with Aβ as an *in vitro* model.

2. Materials and Methods

2.1. Materials. Amyloid beta-protein (25–35) trifluoroacetate salt (Aβ_{25-35}) was provided by Bachem California (Torrance, CA). RPMI+GlutaMAX-l, penicillin-streptomycin, fetal bovine serum (FBS), and horse serum (HS) were purchased from Invitrogen (Grand Island, NY). BCA protein assay kit was purchased from Thermo Fisher Scientific (Barrington, IL). The assay kit for cytotoxicity (WST-8) was supplied by Cayman Chemical Company (Ann Arbor, MI). Lipid peroxidation colorimetric assay kit was obtained from Oxford Biochemical Research (Rochester Hills, MI). SB203580 (p38 inhibitor), SP600125 (JNK inhibitor), and Protoporphyrin IX zinc (II) (Znpp, HO-1 Inhibitor) were obtained from Sigma-Aldrich, Inc. (St. Louis, MO). Antibodies to Nrf2 (C-20) were from Santa Cruz Biotechnology (Santa Cruz, CA); phosphor-SAPK/JNK rabbit mAb, phospho-p44/42 MAPK (ERK1/2) rabbit mAb, phosphor-p38 MAP kinase rabbit mAb, HO-1 (P249) rabbit mAb, glyceraldehydes-3-phosphate dehydrogenase (GAPDH) rabbit mAb, anti-rabbit IgG alkaline-phosphatase- (AP-) linked antibodies, and U0126 (ERK inhibitor) were obtained from Cell Signaling Technology (Danvers, MA). All the other reagents were of the highest grade and were obtained from Sigma-Aldrich (St. Louis, MO), unless otherwise indicated.

2.2. Preparation of Decursin. D was prepared by Dr. Kim's lab in the Department of Smart Foods and Drugs, Inje University, as described previously [52]. Briefly, dried and powdered root of *A. gigas* Nakai (1 kg) was extracted with 5 L of 95% ethanol for 24 h at room temperature. Extracts were filtered through Whatman No. 1 filter paper and were concentrated using a rotary evaporator (R-200, Büchi Labortechnik AG, Flawil, Switzerland) under reduced pressure, and 50 g *A. gigas* Nakai ethanol extract (AGNEX) was obtained. D was purified from AGNEX using recycling preparative HPLC (LC-9104, JAI, Tokyo, Japan). The AGNEX (20 g) was dissolved in 30 mL of 70% acetonitrile/water and filtered with a 0.45 μm membrane filter. 3 mL of sample was injected to the JAIGEL ODS-AP column (20 × 500 mm, JAI) at a flow rate of 4 mL/min. Isocratic elution was applied using 70% acetonitrile/water as the mobile phase, and the peaks were detected using an RI and UV/Vis detector at 328 nm. Finally, 5.3 g of D was obtained.

2.3. Preparation of $A\beta_{25-35}$ Stock Solution. $A\beta_{25-35}$, the most toxic peptide fragment derived from the amyloid precursor protein (APP), was dissolved in deionized distilled water at a concentration of 1 mM and was incubated in 37°C for 3 d to induce maximal aggregation according to the previous report [53]. In order to create stable conditions for the aged stock solution, the solution was stored at −80°C and diluted in serum-free medium to desired concentrations immediately before use.

2.4. Cell Culture. The rat pheochromocytoma (PC12) cell line kindly provided by Dr. Kam (Inje University) was maintained in RPMI+GlutaMAX-l supplemented with 5% FBS, 10% HS, and 1% penicillin-streptomycin and cultured at 37°C in a humidified atmosphere of 5% CO_2. All cells were plated in poly-*L*-lysine coated culture dishes. The medium was changed every other day, and the cells were plated at an appropriate density according to the scale of each experiment. After the 24 h subculture, cells were switched to serum-free medium for treatment.

2.5. Assay for Cell Viability. The cell viability was assessed by the WST-8 cell proliferation assay kit according to the manufacturer's instructions (Cayman Chemical Company, Ann Arbor, MI). Briefly, PC12 cells were seeded in 96-well culture plates. After incubation, the media were supplemented with 10 μL/well WST for 2 h prior to spectrophotometric evaluation. Conversion of WST to formazan was measured at 450 nm by fluorescence multidetection reader (Synergy HT, Biotek, Highland Park, IL). This reaction reflects the reductive capacity of the cells, representing the viability of the cells, and the results were expressed as the percentage of control (untreated) cells. Decreased WST reduction was taken as an indication of neuronal cell injury.

2.6. Assay of Intracellular Reactive Oxygen Species. ROS production in PC12 cells was measured using the redox-sensitive fluorescent dye H_2DCF-DA. Briefly, PC12 cells were seeded in 96-well plates, following treatment, the cells were loaded with 10 μM H_2DCF-DA at 37°C for 30 min in the dark and then washed twice with PBS, and finally, the fluorescence intensity was measured at the excitation wavelength of 485 nm and the emission wavelength of 530 nm using a fluorescence microplate reader (Synergy HT, Biotek, Highland Park, IL). Data were analyzed and expressed as a percentage of the control.

2.7. Assay of HO Activity. The HO activity was measured by spectrophotometric determination of bilirubin formation according to the previously described procedures. Briefly, microsomes obtained from harvested cells were added to a reaction mixture (1 mL final volume, pH 7.4) containing NADPH, bilirubin reductase from rat liver cytosol, and the substrate hemin. The reaction was conducted at 37°C in the dark for 1 h, terminated by the addition of 1 mL of chloroform, and the extracted bilirubin was measured by the difference in absorbance between 464 and 530 nm.

2.8. Nuclear and Cytosolic Lysate Preparation. Cells were treated with various chemicals, as detailed in the figure legends. Nuclear extracts were prepared with a commercial kit according to the manufacturer's instructions (Active Motif, Carlsbad, CA). All steps were carried out on ice or at 4°C unless stated otherwise. Protease inhibitors (10 μg/mL aprotinin, 10 μg/mL leupeptin) and a reducing agent (1 mM dithiothreitol, 1 mM phenylmethyl sulfonyl fluoride) were added to each buffer just prior to use. Briefly, cells were incubated in 5 vol of hypotonic buffer A (20 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, and 10 mM KCl) on ice for 15 min and homogenized. Nuclei were recovered by centrifugation at 3000 ×g for 15 min, and the supernatant was kept as the cytoplasmic extract. The nuclei were washed once using nuclei wash buffer (10 mM HEPES (pH 7.9), 0.2 mM $MgCl_2$, and 10 mM KCl) and extracted for 30 min on ice in buffer C (20 mM HEPES (pH 7.9), 25% glycerol, 420 mM NaCl, 0.2 mM EDTA, and 1.5 mM $MgCl_2$). Insoluble material was removed by centrifugation at 21,000 ×g for 10 min. The supernatant was used as the nuclear extract. All the protein fractions were stored at −70°C until use, and the protein concentrations were measured with a BCA protein assay kit (ThermoFisher Scientific, Barrington, IL).

2.9. Western Blot Analysis. Western blot was performed by the standard method. Equal amounts of proteins were fractionated by 10% SDS-polyacrylamide gel electrophoresis and electrotransferred to an Immun-Blot PVDF membrane (0.2 μM pore size, Bio-Rad). Membranes were blocked overnight at 4°C in Tris-buffered saline (TBS), 0.05% (v/v) Tween-20, 150 mM NaCl, and 5% (w/v) bovine serum Albumin (BSA, Santa Cruz Biotechnology, Santa Cruz, CA), followed by 2 h incubation with primary antibody diluted in the same buffer (Nrf2 1 : 250, HO-1 1 : 1000, phosphor-p42/44 MAPK (ERK1/2) 1 : 1000, phosphor-SAPK/JNK 1 : 1000, phosphor-p38 MAPK 1 : 1000, and GAPDH 1 : 1000). After washing with 0.1% (v/v) Tween-20 in TBS, the membrane was incubated with anti-rabbit IgG AP-linked secondary antibody for 1 h at room temperature and then washed with the same buffer. The immunoblotted membrane was developed with 5-bromo-4-chloro-3-indoyl phosphate (BCIP)/nitro blue tetrazolium (NBT) color-developing solution. The blots in the samples were quantified by densitometry analysis using PDQuest software (version 7.0, Bio-Rad, Hercules, CA). All data from three independent experiments were expressed as the relative intensity compared to the control group for the statistical analyses.

2.10. DPPH-Scavenging Capacities. D and α-tocopherol were evaluated for their activities to scavenge the stable DPPH radical according to a previously described method. The affinity of the test material to quench the DPPH free radical was evaluated according to the equation scavenging % = (Ac − As)/Ac × 100%. As and Ac are the absorbance at 517 nm of the reaction mixture with sample and control, respectively.

2.11. Trolox Equivalent Antioxidant Capacity (TEAC) Analysis. The ABTS radical cation was prepared by mixing an ABTS

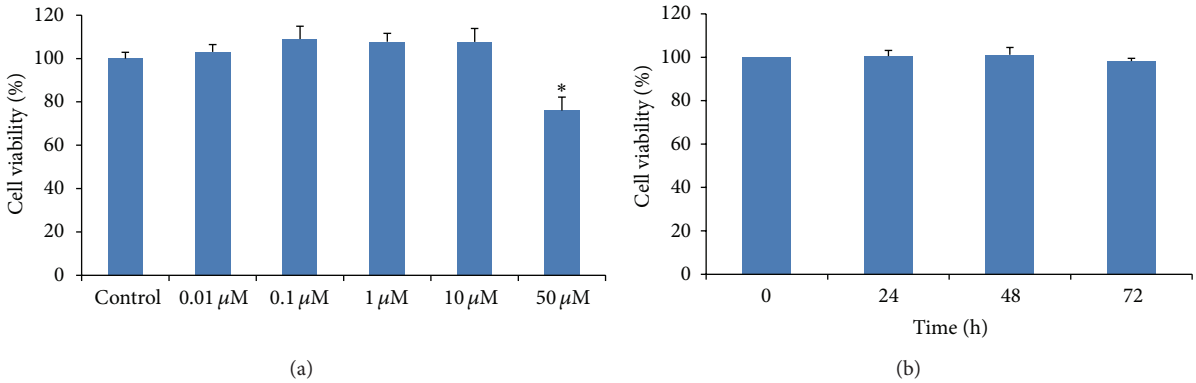

Figure 1: Effect of D on cell viability of PC12 cells. (a) PC12 cells were incubated for 24 h with various concentrations of D (0.01–10 μM). (b) PC12 cells were incubated with 10 μM D for 24, 48, and 72 h. Cell viability was estimated by the WST-8 assay. Data are expressed as percent of values in untreated control cultures and represent the means ± SD for three experiments with triplicates. $^{*}P < 0.05$ compared with control.

stock solution (7 mM in water) with 2.45 mM potassium persulfate. This mixture has to remain for 12–24 h until the reaction is complete and the absorbance is stable. For measurement, the ABTS•+ solution was diluted to an absorbance of 0.700 ± 0.020 at 734 nm. 1 mL ABTS•+ solution and 100 μL of the antioxidant solution were mixed for 45 s and the absorbance at 734 nm was recorded after 1 min of incubation. TEAC is defined as the concentration (mM) of Trolox having the antioxidant activity equivalent to a 1.0 mM concentration of D.

2.12. Statistical Analysis. Data bars represent the means ± SD (standard deviation) for at least three independent experiments in all cases. Two group comparisons were evaluated by student's t-tests as appropriate. Differences were considered statistically significant when P value was <0.05.

3. Results

3.1. Effect of D on Cell Viability of PC12 Cells. Initially, the cytotoxic potential of D on PC12 cells was measured. No cytotoxic effects of D were reported up to a concentration of 10 μM, using the WST assay. However, higher amount D reduced the viability of the PC12 cells (Figure 1(a)). Thus, for further experiments, the cells were treated with D in the concentration range of 0.01–10 μM. Furthermore, 10 μM D treatment for 24, 48, and 72 h did not show any toxic effect on cultured PC12 cells (Figure 1(b)).

3.2. Effect of D on HO-1 Expression and HO Activity of PC12 Cells. As HO-1 is an important component of the cellular defense against oxidative stress, we assessed whether noncytotoxic concentrations (0.01–10 μM) of D affected HO-1 protein expression and HO activity. PC12 cells exposed to D for 24 h caused a dose-dependent increase in HO-1 expression (Figure 2(a)) and HO activity (Figure 2(c)). At a concentration of 10 μM D, HO-1 induction was evident at 3 h, peaked at around 6 h, and decreased after 12 h in PC12 cells (Figure 2(b)). Consistently, D-induced HO activity directly correlated with enhanced HO-1 protein level (Figure 2(d)).

3.3. Effect of D on Aβ_{25-35}-Induced Cytotoxicity and Intracellular ROS Generation in PC12 Cells. To evaluate the *in vitro* neuroprotective effect of D, we tested its protective effect on Aβ_{25-35}-induced cytotoxicity in PC12 cells. PC12 cells were treated with various sub-lethal concentrations of D for 3 h, followed by further incubation for 24 h in the presence or the absence of Aβ_{25-35}. Treatment with Aβ_{25-35} (25 μM) for 24 h induced approximately 40% cell death, whereas D, at non-cytotoxic concentrations (0.01–10 μM), resulted in marked enhancement of survival of the PC12 cells as compared to the Aβ_{25-35}-treated group (Figure 3(a)). Maximal rescue occurred at a concentration of 10 μM of D. These results showed that Aβ_{25-35} treatment significantly reduced the viability of PC12 cells and that D blocked the injury caused by Aβ_{25-35}.

It has been reported that Aβ impairs mitochondrial redox activity and increases the generation of ROS [54–56]. The degree of intracellular ROS generation in cells was measured using fluorescence assay with H_2DCF-DA probe. H_2DCF-DA can be deacetylated in cells, where it can react quantitatively with intracellular radicals, mainly H_2O_2, and convert into its fluorescent products DCF, which are retained within the cell. Therefore, this assay provides an index of cell cytosolic oxidation. As shown in Figure 3(b), treatment with D significantly reduced the Aβ_{25-35}-induced ROS generation, indicating that D attenuates ROS production. Moreover, as shown in Figure 3(c), this effect was dependent on the duration of D pretreatment. Thus, attenuation of ROS released by D required the presence of D at least 3 h prior to the addition of D. These results suggest that D induced the expression of a gene(s) essential to ROS antagonism.

3.4. Effect of HO-1 Expression on Aβ_{25-35}-Induced Oxidative Neurotoxicity Mediated by D in PC12 Cells. Recent reports have described the expression of HO-1 as an adaptive and protective response against oxidative insult in a wide variety of cells, such as PC12 cells. To test whether the protective effect of D is related to its inductive effect on HO-1 expression, we tried to block the activity of HO-1 using Znpp, the inhibitor of HO activity. As shown in Figure 4, D-induced HO-1

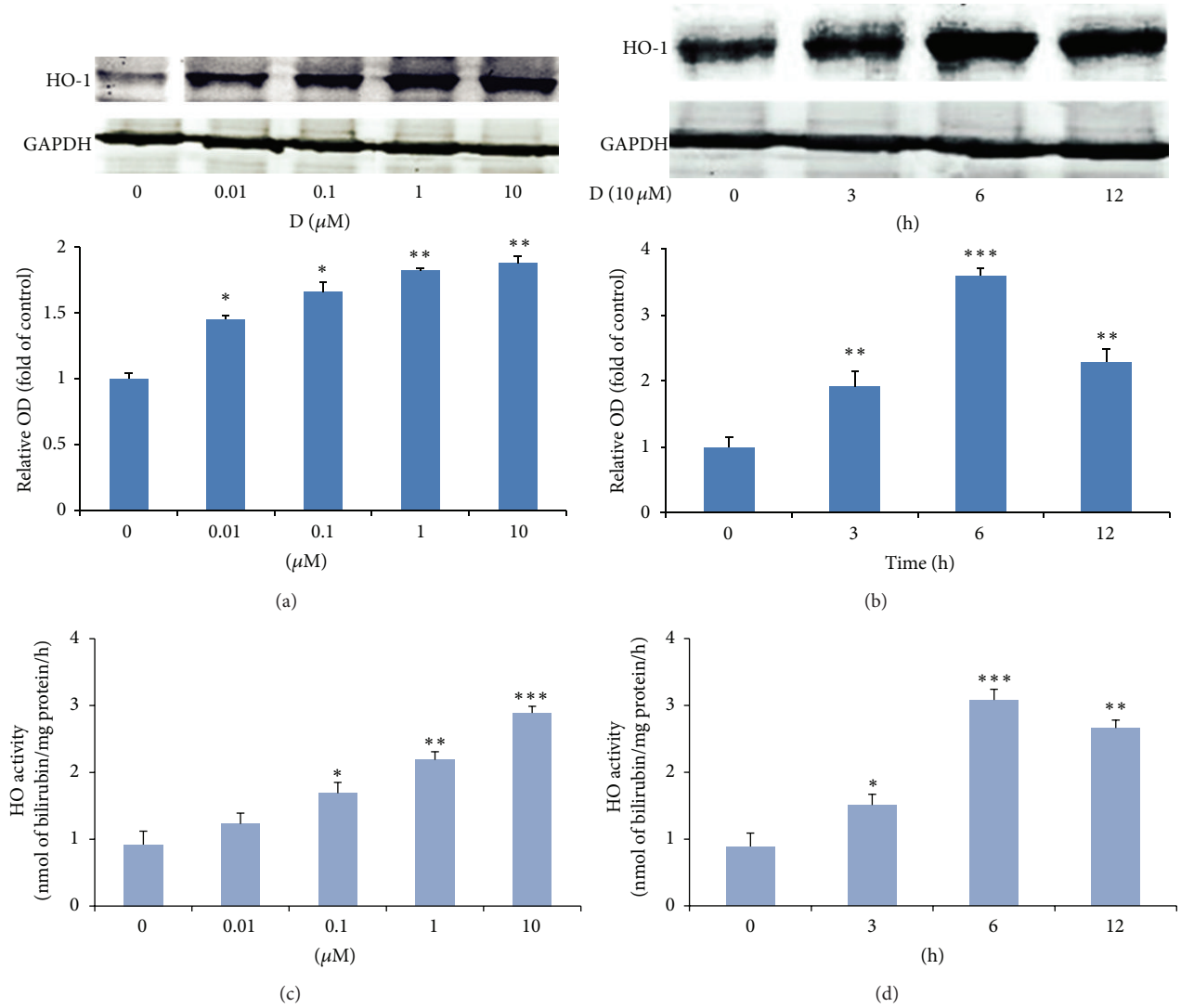

Figure 2: Effects of D on HO-1 expression and HO activity in PC12 cells. (a) Cells were incubated with various concentrations of D for 24 h. (b) Cells were incubated for indicated periods with 10 μM of D. Expression of HO-1 was determined by Western blot analysis, and representative blots of three independent experiments are shown. (c) HO activity was determined *via* bilirubin formation at 24 h after treatment with various concentrations of D. (d) PC12 cells were treated with 10 μM of D, and HO activity was measured at the indicated time points. Each bar represents the means ± SD of three independent experiments with triplicates. $^{*}P < 0.05$ compared with control. $^{**}P < 0.01$ compared with control. $^{***}P < 0.001$ compared with control.

expression was required for suppressing Aβ_{25-35}-induced cell death and ROS generation. The HO-1 inducer CoPP showed comparable protection to D. ZnPP, abrogated the protective effect of D on Aβ_{25-35}-induced cytotoxicity, and partially reversed the inhibitory effects of D on ROS production. These results suggest that the cytoprotective effect of D is mediated through HO-1 induction.

3.5. Effects of D on Nrf2 Nuclear Translocation in PC12 Cells. Several studies have reported that nuclear translocation of activated Nrf2 is an important upstream contributor to the mechanism of HO-1 expression. Therefore, we examined whether D could induce the translocation of Nrf2 to the nucleus in PC12 cells. Using Western blot analysis, we tested the presence of Nrf2 proteins in nuclear compartments of PC12 cells. The cells were incubated with 10 μM D for 0, 1, 3, and 6 h. As shown in Figure 5(a), the nuclear fractions of D-treated PC12 cells showed a gradual increase in Nrf2 levels which was strongly correlated with the increase in HO-1 expression and HO-1 activity (Figure 2), whereas they were decreased concomitantly in the cytoplasmic fractions (Figure 5(b)).

3.6. Involvement of MAPK Pathway in D-Induced HO-1 Expression and Nrf2 Nuclear Translocation in PC12 Cells. MAPK is activated in response to oxidative stress and other various stressors. Several studies have demonstrated that the activation of the MAPK pathways is involved in

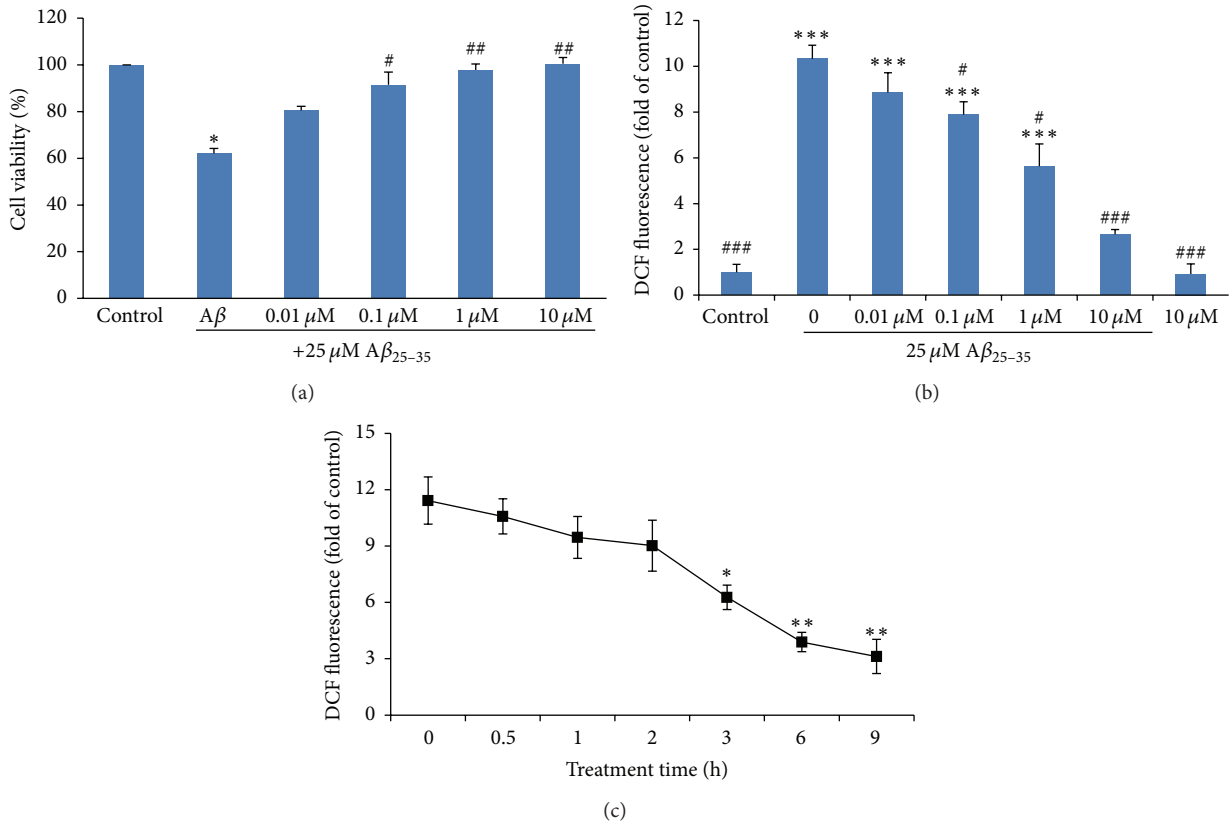

Figure 3: Effect of D on Aβ_{25-35}-induced oxidative neurotoxicity and intracellular ROS generation in PC12 cells. Effect of D on Aβ_{25-35}-induced oxidative neurotoxicity in PC12 cells. (a) PC12 cells were pretreated with various concentrations of D for 3 h and then incubated with and without 25 μM Aβ_{25-35} for 24 h. Cell viability was estimated by the WST-8 assay. Effect of D on Aβ_{25-35}-induced intracellular ROS generation in PC12 cells. (b) PC12 cells were pretreated with various concentrations of D for 3 h and then incubated with and without 25 μM Aβ_{25-35} for 24 h. After that the cells were washed with PBS and then treated with 10 μM H_2DCF-DA for 30 min. (c) PC12 cells were incubated with D (10μM) for the indicated time. They were then treated with 25 μM Aβ_{25-35} for 24 h and with 10 μM H_2DCF-DA for 30 min. Intracellular ROS production was measured at the excitation wavelength of 485 nm and the emission wavelength of 530 nm using the fluorescence microplate reader. Each bar represents mean ± SD from three experiments with triplicates. $^{*}P < 0.05$ compared with control. $^{**}P < 0.01$ compared with control. $^{***}P < 0.001$ compared with control. $^{\#}P < 0.05$ compared with the group treated by Aβ_{25-35} alone. $^{\#\#\#}P < 0.001$ compared with the group treated by Aβ_{25-35} alone.

regulating the translocation of Nrf2 and ARE-mediated HO-1 gene expression [57, 58]. To further elucidate the upstream signaling pathway involved in D-mediated Nrf2 activation and HO-1 induction, we examined the effect of D on activation of MAPKs in PC12 cells. Cells were exposed to D, total protein was harvested, and then Western blots were performed using anti-phospho-JNK, ERK, and p38 antibodies. At a concentration of 10 μM, which strongly induced the levels of HO-1, D caused prolonged ERK activation. As illustrated in Figure 6, phosphorylation of ERK was observed 0.5 h after D treatment and was sustained for up to 1 h after D treatment. In contrast, phosphorylation of p38 kinases was decreased. No changes in the expression of phospho-JNK protein were detected, verifying that similar amounts of proteins were loaded in each lane.

Next, to address the role of MAPK in D-induced HO-1 expression against Aβ_{25-35}, we examined the effects of specific inhibitors of ERK (U0126), JNK (SP600125), and p38 (SB203580) on the levels of HO-1 and cell viability, by Western blot and WST assay. As shown in Figures 7(a) and 7(b), The U0126 significantly reduced D-induced HO-1 expression and activity. Likewise, D against Aβ_{25-35}-induced cell death was effectively abolished by U0126 (Figure 7(c)), whereas p38 inhibitor increased these items. The inhibitor of JNK did not show any changes at any of the tested time periods. Therefore, we suggested that D-induced expression of HO-1 was mediated through ERK pathway phosphorylation and decreased p38 activation in the PC12 cells. U0126, SP600125, and SB203580 alone did not alter cell viability in control or Aβ_{25-35}-treated cells (data not shown).

Furthermore, we examined whether the MAPK pathway was involved in D-induced Nrf2 nuclear translocation. As shown in Figure 7(d), inhibitor of the ERK MAPK pathway blocked D-induced Nrf2 nuclear translocation; on the contrary, p38 inhibitor facilitated the translocation of Nrf2. These results indicate a role for MAPK signaling in D-mediated HO-1 induction through nuclear translocation of Nrf2.

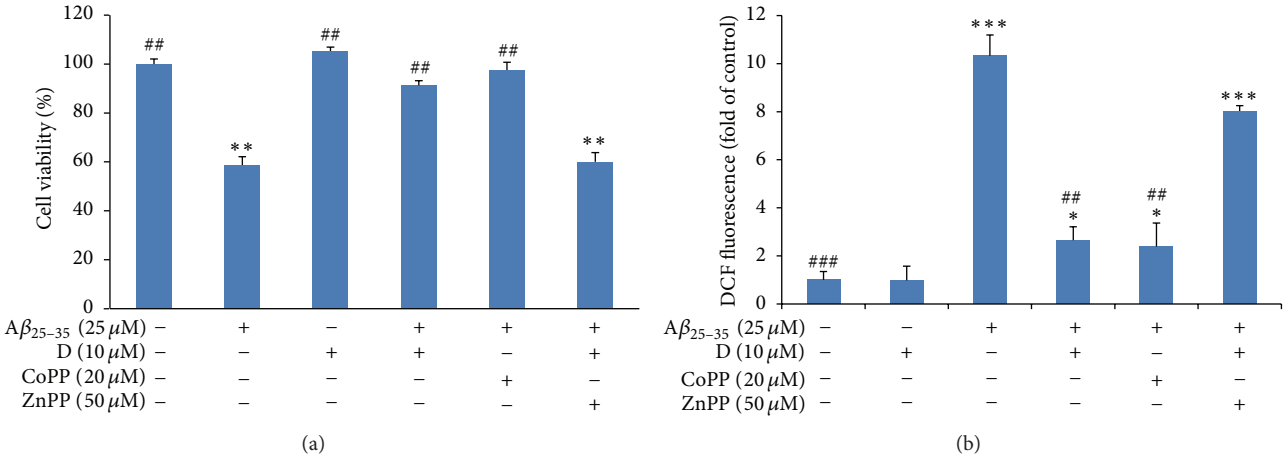

Figure 4: Effect of HO-1 expression on $A\beta_{25-35}$-induced oxidative neurotoxicity mediated by D in PC12 cells. (a) Cells were treated with 10 μM of D or 20 μM CoPP in the presence or absence of 50 μM ZnPP and then exposed to $A\beta_{25-35}$ (25 μM) for 24 h. (b) Exposure of PC12 cells to 25 μM $A\beta_{25-35}$ for 24 h increased ROS production. D-induced HO-1 effectively inhibited ROS production. Each bar represents mean ± SD from three experiments with triplicates. $^{**}P < 0.01$ compared with control. $^{***}P < 0.001$ compared with control. $^{\#\#}P < 0.01$ compared with the group treated by $A\beta_{25-35}$ alone. $^{\#\#\#}P < 0.001$ compared with the group treated by $A\beta_{25-35}$ alone.

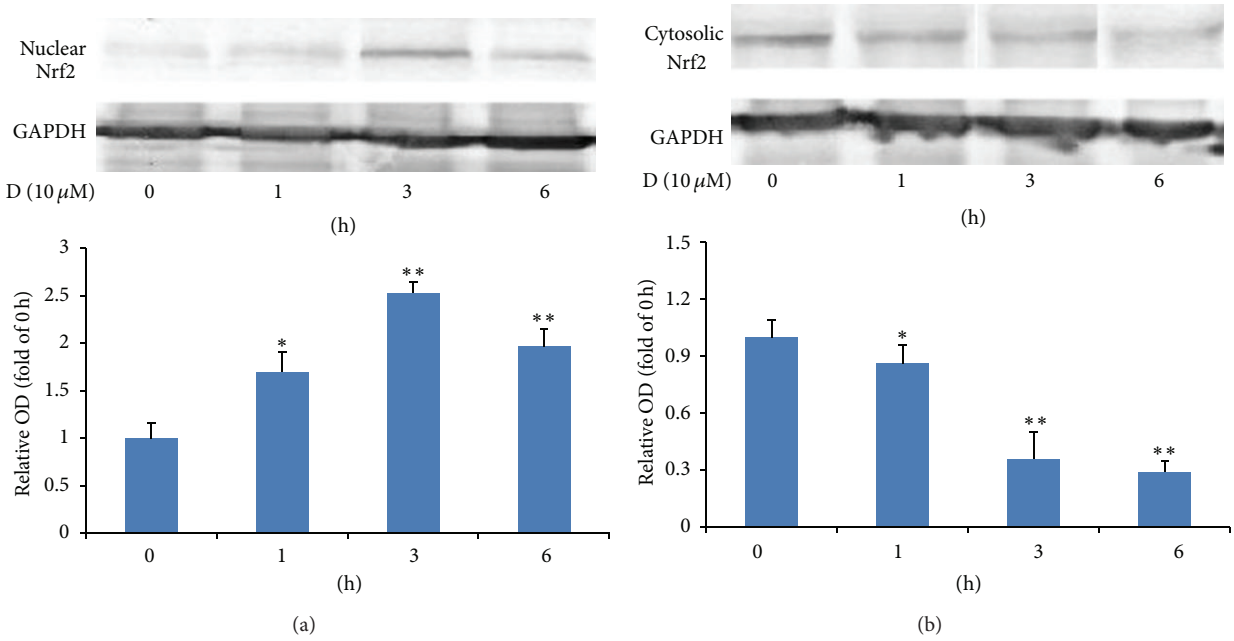

Figure 5: Effects of D on Nrf2 nuclear translocation in PC12 cells. Cells were treated with 10 μM D for 0, 1, 3, and 6 h, after which the nuclear (a) and cytosolic (b) Nrf2 proteins were determined by Western blot analyses. Data shown represent the means ± SD expressed as fold of 0 h group values obtained from three separated experiments with triplicates. $^{*}P < 0.05$ compared with 0 h group. $^{**}P < 0.01$ compared with 0 h group.

3.7. Effect of D on Free Radical Scavenging Activities. To evaluate the antioxidant activity of D, we started by investigating its DPPH-scavenging actions. The DPPH stable free radical method is an easy, rapid, and sensitive way to survey the antioxidant activity of compounds or extracts. Figure 8(a) demonstrates that DPPH-scavenging potentials increased as the concentrations of D and α-tocopherol increased. This result indicates that the antioxidant effect of D is similar to α-tocopherol for trapping DPPH. The TEAC of D was further measured from the decolorization of ABTS•+, which was measured spectrophotometrically at 734 nm. Figure 8(b) shows that D has compatible antioxidant potential with positive control Trolox.

4. Discussion

There is considerable evidence supporting oxidative stress is implicated in the pathogenesis of neurodegenerative disorders such as Alzheimer's disease (AD), cerebral ischemia, and Parkinson's disease (PD) [59, 60]. Several lines of

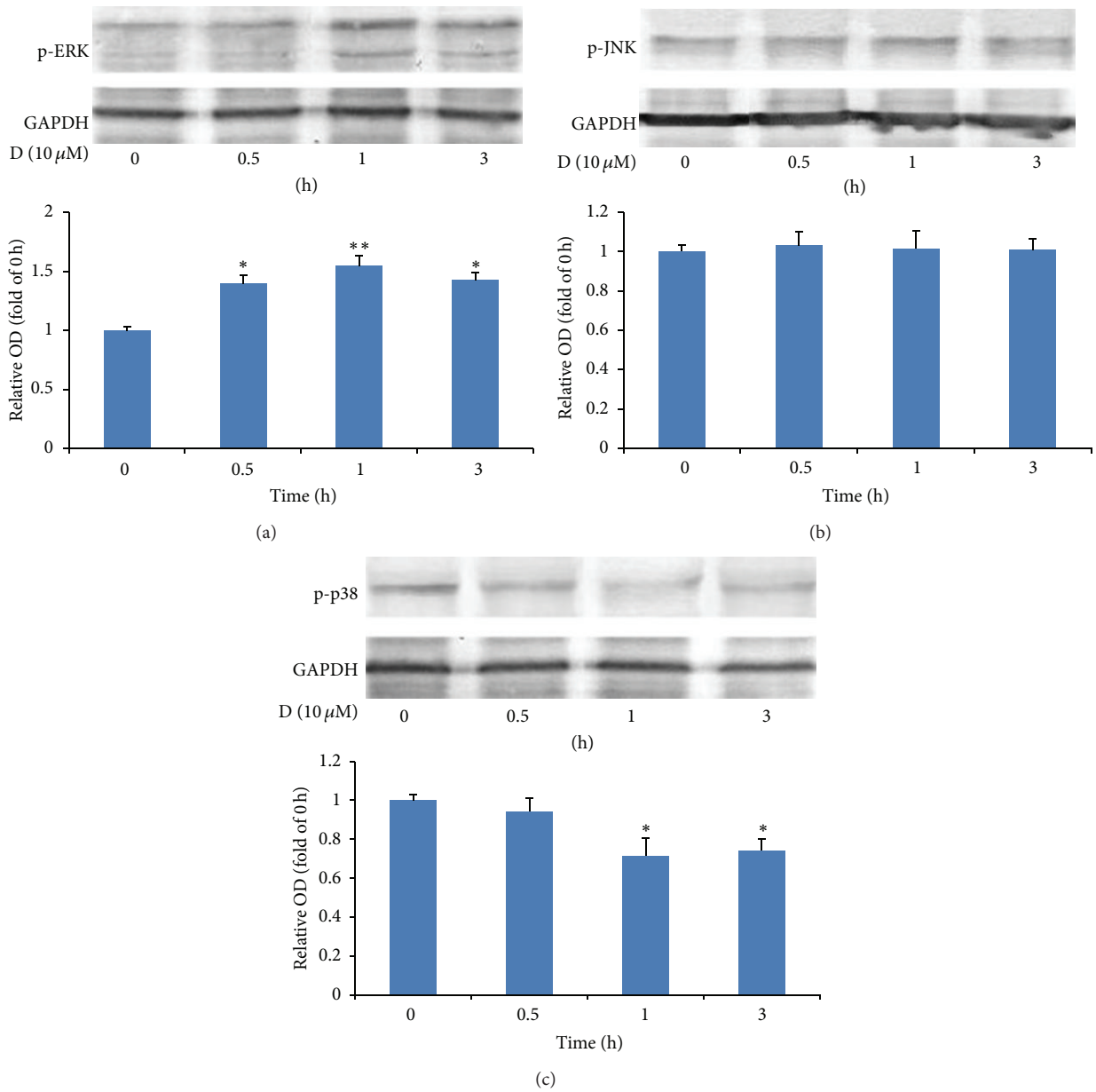

Figure 6: Effects of D on ERK (a), JNK (b), and p38 MAPK (c) in PC12 cells. Cells were treated with 10 μM D for the indicated times. Cell extracts were analyzed by Western blot with antibodies specific for phosphorylated ERK (p-ERK), phosphorylated JNK (p-JNK), or phosphorylated p38 (p-p38). Data shown represent the means ± SD expressed as fold of 0 h group values obtained from three separated experiments with triplicates. $^{*}P < 0.05$ compared with 0 h group. $^{**}P < 0.01$ compared with 0 h group.

evidence indicate that beta amyloid peptide (Aβ) has a causal role in the development and progress of AD. In AD, oxidative stress is suspected to be generated by Aβ [56]. Recent studies showed that there is a vicious circle among Aβ production/accumulation and oxidative stress [61, 62]. Given the important role of oxidative stress in AD, therapeutic strategies which are directed at early interventions targeted at oxidative stress may be effective in delaying Aβ development and slowing its progression. A feasible ways to prevent ROS-mediated cellular damage is to augment the intracellular oxidative defense capacity through dietary or pharmacological intake of antioxidants. Moreover, the induction of endogenous phase II detoxifying enzymes or antioxidative proteins seems to be a reasonable strategy for delaying disease progression and the toxic effects associated with Aβ-mediated cytotoxicity [63–66]. Indeed, increased cellular antioxidant activity confers protection and has been reported to lower the risk of AD [14].

Previous studies have revealed that D could increase cellular resistance to Aβ_{25-35}-induced oxidative injury in PC12 cells. However, the mechanisms by which D mediates its therapeutic effects against Aβ_{25-35}-induced neuronal death *in vitro* remain an interesting speculation that awaits further investigation. Since previous findings support the importance

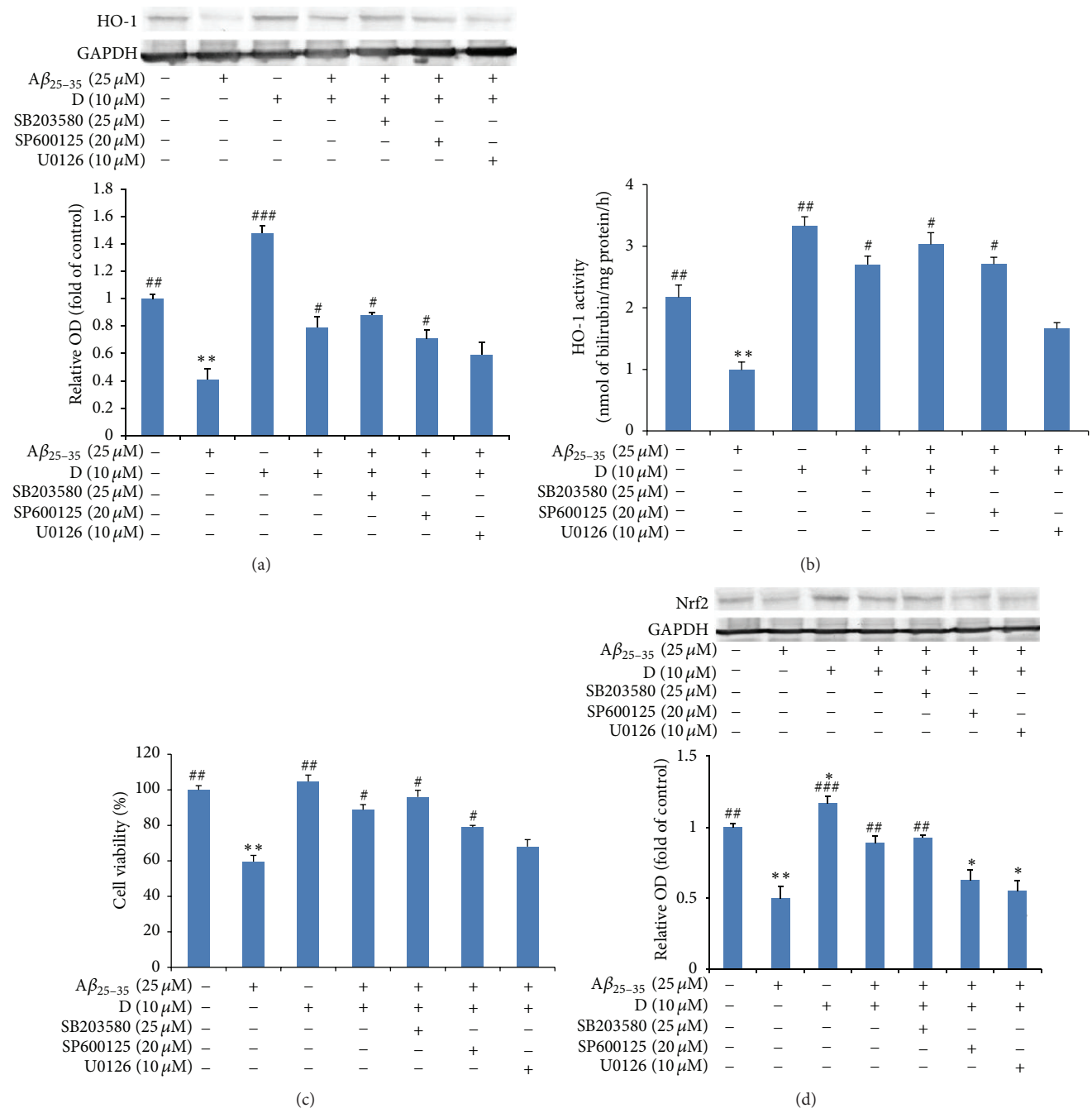

Figure 7: Effects of D-induced MAPK activation on expression (a) and activity (b) of HO-1, neurotoxicity (c), and Nrf2 translocation (d) in $A\beta_{25-35}$-induced PC12 cells. Cells were treated with 10 μM D with and without the inhibitors of MAPK. Cell extracts were analyzed by Western blot with specific antibodies. Each bar represents means ± SD from three experiments with triplicates. $^{*}P < 0.05$ compared with control. $^{**}P < 0.01$ compared with control. $^{\#}P < 0.05$ compared with the group treated by $A\beta_{25-35}$ alone. $^{\#\#}P < 0.01$ compared with the group treated by $A\beta_{25-35}$ alone. $^{\#\#\#}P < 0.001$ compared with the group treated by $A\beta_{25-35}$ alone.

of HO-1 in protection of neurons against Aβ-induced oxidative stress-dependent injury [67], as a part of our continuing research, in this study, we attempted to explain the possible molecular mechanisms underlying the antioxidant effects of D against Aβ-induced oxidative cell death, with focus on upregulation of HO-1 and the underlying regulatory signaling pathways.

The extent and the rapidity of quenching the DPPH free radical and ABTS radical cation are the criteria commonly used to assess relative antioxidant capacity of flavonoid compared with standard antioxidants, α-tocopherol, and Trolox [68, 69]. Current research demonstrates that D has compatible antioxidant potential with positive control, α-tocopherol, and Trolox (Figure 8). These results showed that

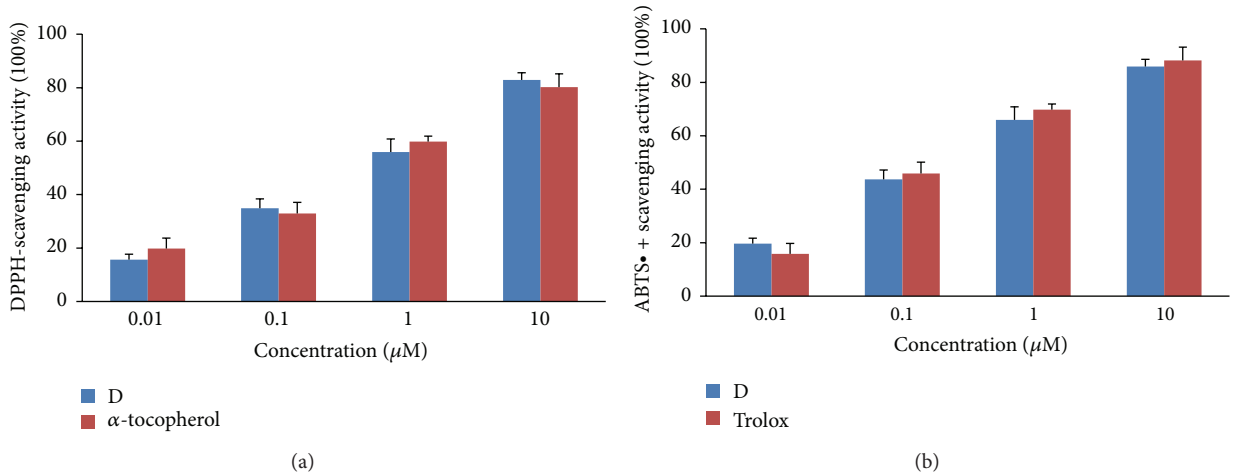

Figure 8: Effects of D on DPPH (a) and ABTS•+ (b) free radical scavenging. Data represent the means ± SD of three independent experiments.

D possesses powerful antioxidant potency, which are consistent with the previous reports showing that D is an oxygen-free radical scavenger. This was also confirmed with our cell-based antioxidant assay in which D significantly reduced the basal intracellular level of ROS (Figure 3). Aβ_{25-35} caused a significant decrease in PC12 cell viability, and pretreatment cells with D concentration-dependently increased cell viability (Figure 4(a)). These results showed a clear and strong correlation among free radical-quenching activities, ROS scavenging activity, and the enhanced resistance to Aβ_{25-35}-induced oxidative damage. As a consequence, the intrinsic antioxidant capacity may play a role for D, in contributing towards the partial or total alleviation of cellular oxidative stress.

HO-1, also known as heat-shock protein 32 (HSP 32) or inducible HO, is a 32-kDa protein transiently activated by a wide variety of noxious stimuli including oxidative stress. Upregulation of HO-1 leading to elevation of HO activity has been shown to provide neuroprotective effects by converting the prooxidant heme to biologically active antioxidant by-products such as biliverdin/bilirubin and also to heme inactivating carbon monoxide. The transgenic mice overexpression HO-1 in the brain attenuated neuronal cell injury caused by ischemic stroke [70]. HO-1 overexpressing cells derived from transgenic mice or the cells treated with a HO-1 inducer are relatively resistant to oxidative stress [20, 22]. Induction of HO-1 is highly recognized as an important therapeutic target for pharmacological intervention of oxidative disorders. It has been suggested that an elevation of HO-1 by various stimuli may be protective cellular response to delay the cell death. Therefore, we are interested in determining the potential role of HO-1 in the Aβ_{25-35}-induced PC12 cells damage and the D-mediated neuroprotection. We have provided evidence for the induction of HO-1 by D in PC12 cells and showed that D-induced HO-1 protein expression and HO activity occurred in a concentration- and time-dependent manner (Figure 2). Furthermore, the increase of HO-1 expression by D conferred cytoprotection against Aβ_{25-35}-induced oxidative stress (Figure 3). In addition, we showed that ZnPP, a potent inhibitor of HO activity, can partially reverse the protective effects of D, thus providing further evidence for HO-1 as a possible cytoprotective pathway for D (Figure 4(a)). The induction of HO-1 expression was also required to suppress Aβ_{25-35}-induced ROS generation (Figure 4(b)). These results strongly indicate that in our experimental setting, D may have multiple mechanisms of action that affect cytoprotection both by reducing ROS generation and boosting HO-1 induction for ROS detoxification.

The central sensor of intracellular oxidative stress is the cytosolic Keap1-Nrf2 complex. In response to oxidative stress, Nrf2 is released from Keap1 and transmits the stress signal to the nucleus for activation of distinct set of genes encoding phase II detoxifying enzymes as well as several stress responsive proteins including HO-1 [71, 72]. Recently, Nrf2-ARE signaling pathways have emerged as key therapeutic targets for treatment of a variety of oxidative stress-related neurodegenerative insults [73]. Nrf2-null mice resulted in a decrease in the basal expression level of detoxifying or antioxidant genes including HO-1 [74]. In contrast, Nrf2$^{+/+}$ mice protect the brain from cerebral ischemia *in vivo* [75], whereas primary neuronal cultures treated with chemical activators of the Nrf2-ARE pathway displayed significantly greater resistance to oxidative stress-induced neurotoxicity [73]. Phytochemicals including sulforaphane, caffeic acid phenethyl ester (CAPE), and curcumin activate the Nrf2-ARE system [76, 77]. Using Western blot, we examined whether D activated Nrf2 in PC12 cells and found that Nrf2 was promoted translocation into the nucleus in PC12 cells exposed to a nontoxic concentration of D (Figure 5). The translocation of Nrf2 into the nucleus following D treatment was associated with a marked increase in HO-1 induction (Figure 2). Therefore, our results suggest that the transcriptional activation of Nrf2 is involved in the increased expression of HO-1 and the cytoprotection against Aβ_{25-35} induced in PC12 cells (Figure 7(c)).

The upstream signaling pathways regulating Nrf2 transactivation remain poorly defined. Recent studies have implicated a major role for the MAPK in Nrf2-dependent translocation and HO-1 activation, although other kinases including tyrosine kinases, PI3K, and PKC have also emerged as potential contributing mechanisms coordinately or separately [78, 79]. In vertebrates, the three major kinase cascades are represented by ERK, JNK, and p38 MAPK [80]. The current experiments were designed to verify a possible role of MAPK pathway in D-induced Nrf2 activation and HO-1 expression, and D was found to facilitate the phosphorylation of ERK and dephosphorylation of p38 (Figure 6). In addition, upregulation of HO-1 as well as Nrf2 nuclear translocation by D was remarkably inhibited by U0126, a highly selective inhibitor of ERK pathway. These investigations suggested that the activation of ERK pathway by D contributed to HO-1 expression (Figures 7(a) and 7(b)), Nrf2 nuclear translocation (Figure 7(d)), and cytoprotection (Figure 7(c)) in PC12 cells. It is generally acknowledged that MAPK can be differentially regulated by the same stimuli in diverse cell types. ERK pathway is thought to mediate cellular responses to growth and differentiation factors, whereas JNK and p38 pathway is activated by distinct and overlapping sets of stress-related stimuli [81]. For instance, quercetin induces Nrf2 nuclear translocation and HO-1 upregulation *via* the ERK and p38 pathway but not the JNK pathway in human hepatocytes [82]. 3H-1, 2-dithiole-3-thione increases nuclear Nrf2 accumulation *via* the ERK pathway but not the p38 and JNK pathway in murine keratinocytes [83]. In PC12 cells, 15-Deoxy-D12, 14-prostaglandin J2 induces HO-1expression *via* the ERK and Akt/PI3K pathway but not the p38 and JNK pathway [84]. Shen et al. [79] have shown that the transcriptional activity of Nrf2 transactivation domain was stimulated by ERK and JNK signaling pathways while the p38 MAPK plays a negative role. This may be due to the diverse assortment and intensity of the signaling pathways activated by different inducers in different cell types. However, further studies are extensively ongoing to define the exact role of the MAPK pathways in HO-1 expression.

In summary, the intrinsic free radical scavenging activity and inducing the upregulation of HO-1 expression through activation of Nrf2 exposed to D confer protection against the Aβ_{25-35}-induced oxidative damage in PC12 cells. One of the most salient features of our present study is that ERK is involved in HO-1 induction *via* Nrf2 activation in the D-stimulated cells. Thus, pharmacological inhibition of ERK suppressed Nrf2 activation and subsequent HO-1 expression. Results of our study imply the potential involvement of these upstream kinases in Nrf2 activation and HO-1 upregulation by D. However, the complete molecular milieu that links all these events needs to be elucidated. Continued attempts to identify novel target molecules responsible for the HO-1 regulation and to clarify their cross-talk with upstream and downstream signaling molecules will pave the way to exploiting preventive and/or therapeutic strategies for the management of oxidative stress-mediated disorders. Moreover, considering that the D has a property that can cross the blood-brain barrier, further *in vivo* study with D should substantiate this therapeutic potential of this compound. These findings indicate that D might prove to be a promising therapeutic approach to combat neural demise in AD and other oxidative stress-related diseases.

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