ABSTRACT

Epidemiological studies have strongly linked postmenopausal estrogens uptake with a reduced risk of developing Parkinson’s disease (PD) in women. Estrogen replacement therapy may be beneficial in early PD. The aim of this study is to evaluate the hypothesis that the neuroprotective effects of phytoestrogen β-ecdysterone (β-Ecd) might mainly result from its antioxidant capability by the activation of the phosphoinositide-3-kinase (PI3K)-nuclear factor E2-related factor 2 (Nrf2)-regulated signaling pathway. We found that β-Ecd is able to protect MPP⁺-induced oxidative stress and apoptosis in PC12 cells in a concentration-dependent manner. β-Ecd increased the Akt kinase activity and the Akt signaling pathways, including glycogen synthase kinase 3-β inactivation, nuclear translocation of Nrf2, upregulation of HO-1 expression, but without affecting activity of both NF-κB and calpain. Enhancement of antioxidant response element (ARE) promoter-driven luciferase activity by β-Ecd correlated with the blockade of oxidative stress. Antioxidative effects of β-Ecd could be blocked by pharmacologic inhibition of the PI3K pathways with LY294002 or Nrf2 pathway with shRNA-mediated knockdown of Nrf2 but not by SP600125 (JNK inhibitor), SB203580 (p38-MAPK inhibitor), or PD98059 (ERK1/2 inhibitor). Together, our results indicate that the inducible effect of β-Ecd on HO-1 expression might be mediated, at least in part, by activating Akt kinase pathway and subsequent enhancement of Nrf2/ARE signaling pathway. In concert, these data suggest that β-Ecd may be a potential candidate for further preclinical study aimed at the treatment of PD.

Key words: β-ecdysterone; Parkinson’s disease; oxidative stress; Nrf2; apoptosis.
that Nrf2-deficient mice are hypersensitive to PD-causing toxins, whereas upregulation of Nrf2 either by genetic means or through pharmacological activation renders a neuroprotective response (Kaidery et al., 2013; Lastres-Becker et al., 2012). For this reason, Nrf2 may be considered a therapeutic target for PD that is known to involve free radical damage (reviewed by Cuadrado et al., 2009). Although the causal relationship remains unknown, serine/threonine protein kinase (Akt) activation has been shown to be one of the major upstream events resulting in the activation of Nrf2 and induction of Nrf2 downstream targets (Chen et al., 2012).

Dopamine replacement therapies for PD are mainly targeted at masking or reducing disease symptoms (Maier et al., 2014). There is little evidence indicating that levodopa alters the natural history of the disease or improve survival of the patients (reviewed by Salat and Tolosa, 2013). As such, new disease modifying therapies are needed to address the inexorable progression of PD. Based on a retrospective review of the historical role of a number of Chinese herbal medicine used for the treatment of PD, it was shown that the Achyranthes bidentata Bl. As a component of traditional Chinese medicine prescriptions might potentially provide natural treatment for PD. β-Ecdysterone (β-Ecd; for its structure, see Fig. 1A), a phytoestrogen derived from the root of A. bidentata Bl., has been reported to possess antiapoptotic and anti-inflammatory pharmacological effects (Zhang et al., 2014). No report is available for β-Ecd as an antioxidant to halt the progression of PD. It is so far unknown whether β-Ecd possess potent antioxidant properties that may contribute to their neuroprotective effects, if so, what would be the signaling pathway involved. To address these issues, we hypothesize that β-Ecd inhibits 1-methyl-4-phenylpyridinium (MPP⁺)-induced apoptosis of PC12 cells by interrupting phosphatidylinositol-3-kinase (PI3K)-Nrf2 regulated pathway through attenuating oxidative stress. Indeed, Wang et al. (2011) give the first evidence that this hypothesis holds true. Our findings presented in this study perfectly support the hypothesis.

MATERIALS AND METHODS

Cell culture and treatment. PC12 cells (high differentiation) obtained from the cell bank of Institute of Biochemistry and Cell Biology, SIBS, CAS (Shanghai, China) were cultured at 37°C with
5% CO2 in the medium (DMEM supplemented with 10% FBS and 2 mM L-glutamine). PC12 cells were exposed to MPP+ in the presence or absence of β-Ecd. In some experiments, cells were cotreated with LY294002 to assess if PI3K pathways play a role.

Assessment of cell viability. The number of viable cells was used to determine cell growth by using CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay kit (Promega, Madison, Wisconsin) (ie, MTS assays) with a colorimetric method.

Detection of apoptosis by flow cytometry. Apoptosis was determined by FACSCalibur flow cytometer (BD Biosciences, San Jose, California) and FlowJo software (Tree Star, San Carlos, California) with the Annexin V-FITC/propidium iodide (PI) apoptosis detection kit (KeyGEN Biotech, Nanjing, China) according to the manufacturer’s instructions.

DNA fragmentation. Cytoplasmic histone-bound DNA fragments generated during apoptosis was measured by Cell Death Detection ELISA Plus Assay Kit following the manufacturer’s instructions (Roche Molecular Biochemicals, Indianapolis, Indiana), and the absorbance at 490 nm was determined with a microplate reader (BioTek ELX800, BioTek Instruments Inc., Vermont).

Cell-cycle analysis. Cell-cycle phase analysis was performed by PI staining using FACSCalibur flow cytometer (BD Biosciences) and ModFit LT software (Verity Software House, Topsham, Maine).

Lactate dehydrogenase (LDH) assay. LDH Cytotoxicity Detection Kit (Takara, Shiga, Japan) was used for the quantification of cell damage by measuring LDH release from PC12 cells according to the manufacturer’s protocol. Data were expressed as a percentage of the total LDH release, which was obtained by adding 2% Triton X-100 (Solarbio, Beijing, China) to untreated cells.

Measurement of ROS. Total cellular ROS activity was measured using OxiSelect Intracellular ROS Assay Kit (Cell Biolabs, Inc., San Diego, California) according to the manufacturer’s protocol and the fluorescence was read with a microplate reader (Safire2, Tecan Group Ltd, Maennedorf, Switzerland) at 480/530 nm.

Assessment of glutathione (GSH)/oxidized glutathione (GSSG) ratio. The levels of GSH and GSSG were measured using the
GSH/GSSG-Glo Assay Kit (Promega) according to the manufacturer’s protocol.

NAD⁺/nicotinamide adenine dinucleotide diaphorase (NADH) assay. The NAD⁺ and NADH levels were measured using the NAD⁺/NADH Quantification Kit (BioVision Inc., Milpitas, California), following the manufacturer’s recommendations.

Lipid hydroperoxides (LPO) assays. Hydroperoxide level was determined by using the components provided in Lipid Hydroperoxide Assay kit (Cayman Chemical Company, Ann Arbor, Michigan) according to the manufacturer’s instructions, and concentration was calculated according to the equation in the protocol.

Ferric reducing ability of plasma (FRAP) assay. The FRAP of the β-Ecd (0.4 mM) was determined using a Total Antioxidant Capacity Assay Kit with FRAP method (Beyotime Biotechnology, Haimen, China) according to the manufacturer’s protocol. The results were expressed as relative total antioxidant capacity, which were calculated using on the standard curve obtained using FeSO₄ in the concentration range of 0.15–1.5 mM.

RNA interference. PC12 cells were cultured at 90% confluence in 6-well plates and shRNA plasmid or Control shRNA Plasmid-A (Santa Cruz Biotechnology, Santa Cruz, California) were transfected by FuGENE 6 Transfection Reagent (Promega) according to the manufacturer’s instructions.

Quantitative real-time RT-PCR. Total cellular RNA was isolated using the TRIzol method (Invitrogen, Carlsbad, California). Complementary DNA (cDNA) was synthesized using the Superscript III first-strand synthesis kit (Invitrogen) with 1 μg of total RNA. Quantitative real-time PCR was performed using a SYBR Green PCR Kit (CWBio, Beijing, China) and the following primers: Nrf2: (F) 5'-GCC AGC ACA TCC AGA CAG AC-3'; (R) 5'-TAT CCA GGG CAA GCG ACT C-3'; heme oxygenase-1 (HO-1): (F) 5'-TGC TGG CAT GAA CAC TCT G-3'; (R) 5'-TCC TCT GTC AGC AGT GCC T-3'; glyceraldehyde-3-phosphate dehydrogenase (GAPDH): (F) 5'-GAC ATT TTC GGT ATC GTG GA-3'; (R) 5'-ATG CAG GGA TGA TGT TCG GG-3'. Comparative Ct method was used to calculate the relative fold changes in gene expression normalized against the GAPDH.

Akt kinase assay. Cells were lysed and Akt kinase assay was carried out as per the manufacturer’s instructions by an Akt Kinase Assay Kit (Nonradioactive) purchased from Cell Signaling Technology (Beverly, Massachusetts). In brief, Akt was immunoprecipitated with an immobilized anti-Akt antibody from equal amounts of whole cell extracts and incubated with a glycogen synthase kinase 3 (GSK-3) fusion proteins in the presence of ATP. The phospho-GSK-3α/β(Ser21/9) was detected by immunoblotting.

The activity of nuclear factor κB (NF-κB). NF-κB activity was estimated using enzyme-linked immunosorbent assay (ELISA)-based TransAM NF-κB p65 Assay Kit (Active Motif, Carlsbad, California) on nuclear extracts according to the manufacturer’s protocol, and the absorbance at 450 nm was determined with a microplate reader (BioTek ELX800).

Measurement of calpain activity. Calpain activity was quantified by using a commercially available Calpain Activity Fluorometric Assay Kit (Biovision, Mountain View, California) according to the manufacturer’s instructions, and fluorescence was measured at 400/505 nm with a microplate reader (Safire2).

Luciferase assays. Semi-confluent PC12 cells were transiently transfected with pARE-luc co-reporter plasmid (Beyotime Biotechnology) by using FuGENE 6 Transfection Reagent (Roche Diagnostics, Indianapolis, Indiana). Transfection efficiency was controlled by co-transfection of Renilla luciferase reporter plasmid pRL-TK (Promega). Luciferase activity was measured using a Dual-Glo Luciferase Assay System (Promega) with a microplate reader (Safire2), following the protocol provided by the manufacturer.

Western blotting analysis. Cellular cytoplasmic and nuclear fractions were obtained by using Nuclear and Cytoplasmic Protein Extraction kit (Beyotime Biotechnology) according to the manufacturer’s instructions. Protein was resolved by SDS-PAGE, transferred to nitrocellulose, and probed using primary antibodies [p-Akt, p-GSK-3β, phospho-extracellular signal-regulated kinases 1 and 2 (ERK1/2), HO-1, Nrf2, Akt, GSK-3β, ERK1/2, GAPDH, or Lamin B], which were obtained from Cell Signaling Technology (Beverly, Massachusetts) and appropriate horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology). Protein bands were visualized by the enhanced chemiluminescence light method (Amersham Biosciences, Piscataway, New Jersey).

Statistical analyses. Values are expressed as means ± standard deviation (SD). Differences among means were assessed by 1-way analysis of variance (ANOVA) followed by Student–Newman–Keuls (SNK) post hoc test using the SPSS 13.0 software (SPSS Inc., Chicago, Illinois). Statistical significance was accepted for P values <.05.

RESULTS

β-Ecd Protects PC12 Cells From MPP⁺-Induced Apoptosis

To identify the potential neuroprotective effect of β-Ecd, proliferation of PC12 cells was measured by MTS. Five hundred micromole MPP⁺ were selected based on the previous studies (Wang et al., 2014). As shown in Figures 1B and 1C, β-Ecd increased cell viability in PC12 cells exposed to MPP⁺ in concentration- and time-dependent fashion. Maximal cytoprotective effect was observed at 10 μM for 24 h. Given this result, the treatment of PC12 cells with β-Ecd at 10 μM for 24 h was selected for our further some experiments. To examine the mechanism whereby β-Ecd promotes cell proliferation, the cell cycle was analyzed using a flow cytometry. However, as shown in Figures 1D and 1E, we could not find any significant change in the percentages of S-phase cells after β-Ecd treatment.

LDH activity, an established marker of the loss of cell membrane integrity, was determined in the culture supernatant to examine if protective effect of β-Ecd against MPP⁺-induced cell death was mediated by necrosis of PC12 cells. As shown in Figure 1F, a slight but not significant alteration of LDH activity due to β-Ecd treatment could also be observed in PC12 cells exposed to MPP⁺. Because β-Ecd failed to influence the increase of LDH activity induced by MPP⁺, we hypothesized that the anti-apoptotic property of β-Ecd might play a critical role in prevention of MPP⁺-induced cell death. Annexin V staining followed by flow cytometric analysis showed that apoptosis induced by MPP⁺ was attenuated after β-Ecd treatment (Figs. 1G and 1H).
Consistent with this Annexin V staining, DNA fragmentation analysis also demonstrated that β-Ecd protects against MPP⁺-induced apoptosis in PC12 cells (Fig. 1I).

β-Ecd Suppress MPP⁺-Induced Intracellular Oxidative Stress
To study if antioxidant property of β-Ecd plays a critical role in prevention of MPP⁺-induced apoptosis, we evaluated the antioxidant capability of β-Ecd in PC12 cells exposed to MPP⁺. Our results showed that the pre-exposure of PC12 cells to β-Ecd partially but significantly reduces the intracellular ROS formation caused by MPP⁺ (Fig. 2A). Similar to the results obtained from DCFH-DA assay for cellular ROS, our data from LPO assays showed that β-Ecd significantly blocks MPP⁺-induced increases of LPO in PC12 cells (Fig. 2A). These results suggest that β-Ecd has antioxidant properties.

Usually, GSH exists mainly in the reduced form whereas the oxidized disulfide form (GSSG) is present in small amounts. The ratio of reduced/oxidized GSH is widely regarded as an indicator of oxidative stress (Sentellas et al., 2014). As shown in Figure 2B, β-Ecd partially reverses MPP⁺-induced reduction in GSH-to-GSSG ratio. Given that cytosolic-free NAD⁺/NADH ratio plays a very important role in maintaining cellular redox homeostasis (reviewed by Ying, 2008), we further confirmed antioxidant capability of β-Ecd by the measurements of NAD+/NADH ratio. Our results showed that MPP⁺-mediated depression of NAD+/NADH ratio is significantly elevated by β-Ecd (Fig. 2C), which is consistent with the above-mentioned other oxidative-stress-related biomarkers assay findings. FRAP assay also showed that the β-Ecd exhibited a high antioxidant activity, and the values of the relative total antioxidant capacity are 1.89 ± 0.15.

Nrf2 Nuclear Accumulation Is Essential for β-Ecd Antioxidative Effects
Since ARE regulates the expression of a number of antioxidative enzymes and scavengers, which contribute to the endogenous defense against oxidative stress (reviewed by Nguyen et al., 2009), next we tested if β-Ecd increases ARE promoter activity. As shown in Figure 3A, β-Ecd significantly increases, as expected, the activity of the ARE promoter in PC12 cells after MPP⁺ treatment. However, we observed that ARE promoter activity were marginally but significantly elevated in PC12 cells exposed to MPP⁺ alone, which is contrary to our initial expectation. It has been shown that ARE-driven gene expression, including (but not limited to) HO-1, is regulated mainly by Nrf2. Therefore, we were interested to examine the effects of β-Ecd on Nrf2 and HO-1 genes expression. As shown in Figures 3B–E, β-Ecd augmented MPP⁺-induced increase in both Nrf2 nuclear

![Figure 2. β-Ecd suppress the PC12 cells from MPP⁺-induced oxidative stress. PC12 cells were treated with 1, 3, and 10 μM β-Ecd for 24 h in the presence MPP⁺. A, ROS generation was measured by the cell-permeable fluorogenic probe DCFH-DA with fluorometric method (open bars). LPO level was measured utilizing the redox reactions with ferrous ions by colorimetric method (solid bars). B, Levels of total intracellular GSH (open bars) were determined on the basis of the content of reduced GSH plus GSSG, and ratios of GSSG versus GSH were calculated (solid bars). C, NAD⁺ (open bars) and NADH concentrations were measured by using a Quantification Colorimetric Kit, and ratios of NAD⁺ versus NADH were calculated (solid bars). * P < .05 versus other treatment groups (A), or MPP⁺ alone treatment (B and C); §, P < .05 versus control (B and C).]
mediated inhibition of MPP+ significantly reversed, as expected, the MPP+ severely inhibited by the LY294002 in PC-12 cells exposed to MPP+. The activation of Akt to be a prerequisite for the activation of ARE by 

To address the role of individual mitogen-activated protein kinases (MAPK) and PI3K pathways in activation of ARE by 

Activated Akt Is Involved in β-Ecd-Induced Nrf2 Accumulation

To understand whether Nrf2 genes play a role in prevention of MPP+-induced oxidative stress and apoptosis by β-Ecd. Knockdown of Nrf2 in PC12 cells exposed to MPP+ completely aboli and antioxidant effects of β-Ecd (Fig. 3F), suggesting that Nrf2 is specifically involved in neuroprotective actions of β-Ecd.

β-Ecd Has No Effect on the Both NF-κB and Calpain Activation in PC12 Cell Exposed to MPP+

NF-κB that is often referred to as the cellular ‘sensor’ for oxidative stress is central to regulating apoptosis and cell survival in neurons. To assess the effects of β-Ecd on NF-κB activation, NF-κB activity assay was performed on nuclear extracts of PC12 cells. To our surprise, however, no regulatory effect of β-Ecd on NF-κB activity in PC12 cell exposed to MPP+ could be observed (Fig. 5). Calpain may play a fundamental role in causing neuronal death downstream of mitochondrial oxidative stress and dysfunction (Yamada et al., 2012). Thus, the next question is whether calpain activity is inhibited in PC12 cell exposed to MPP+ following β-Ecd treatment. β-Ecd failed to influence the increase of cellular calpain activity induced by MPP+ (Fig. 5). Overall, these results suggest that the neuroprotective effects of β-Ecd on MPP+-induced injury are unrelated to both calpain and NF-κB pathway.

DISCUSSION

Structurally unique natural products are valid starting points for drug discovery (Tu and Yan, 2012). The discovery of novel mechanisms of action is a vital task for pharmacotherapy (reviewed by Wroński, 2014). The key findings made in this

FIG. 3. Nrf2/ARE signaling is responsible for antioxidative effects of β-Ecd. PC12 cells were treated with 1, 3, and 10 µM β-Ecd for 24 h in the presence MPP+. A, PC12 cells were transiently transfected with the pARE-Luc reporter construct and pRL-TK vector, luciferase activity was determined with the commercial luciferase reporter assay system, and the results expressed as the ratio of firefly/Renilla. B, Cellular cytoplasmic and nuclear fractions were analyzed by Western blotting using antibodies against Nrf2 and HO-1. GAPDH or Lamin B served as loading controls. C, Bar graph shows quantification of nuclear Nrf2 normalized to GAPDH (open bars), and cytoplasmic Nrf2 normalized to Lamin B (solid bars). D, Bar graph shows quantification of HO-1 normalized to GAPDH. E, Real-time RT-PCR was performed and relative expression levels of Nrf2 (open bars) and HO-1 (solid bars) mRNA are normalized against GAPDH. F, ROS generation was measured by the cell-permeable fluorogenic probe DCFH-DA with fluorometric method (open bars). Cytoplasmic histone-associated-DNA-fragments were measured by ELISA assay (solid bars). Values are means ± SD (n = 5). *, P < .05 versus control; †, P < .05 versus MPP+ alone treatment.

Localization and HO-1 expression at levels of transcription and translation. We used an RNAi-based knockdown approach to knockdown endogenous Nrf2 by a RNAi-based knockdown approach and/or block Akt by the PI3K inhibitors LY294002. As shown in Figs 4E and 4F, LY294002 was able to abolish all inactivation of GSK-3β (ie, phosphorylated) and upregulation of Nrf2 and HO-1 expression mediated by β-Ecd. Furthermore, inhibition of Nrf2 by shNrf2 failed to block both β-Ecd-mediated Akt and GSK-3β phosphorylation (Fig. 4G), indicating that Akt phosphorylation is located upstream of Nrf2 nuclear localization.

Considering that Akt and Nrf2 are part of the same antioxidative signaling pathway (Li et al., 2013), we were interested in mapping the chronology between them. To achieve this, we silence endogenous Nrf2 by a RNAi-based knockdown approach of ERK1/2, did not diminish the β-Ecd-mediated ARE activation in PC12 cells (Fig. 4A), indicating that β-Ecd-mediated ARE activation was PI3K-dependent. Akt kinase assay showed that β-Ecd significantly reversed, as expected, the MPP+-induced reduction in Akt kinase activity measured by phosphorylation of GSK-3α/β (Figs 4B and 4C). Blocking PI3K by LY294002 abolished β-Ecd mediated-inhibition of MPP+-induced ROS generation (Fig. 4D), suggesting that activation of Akt is an upstream event that involved in antioxidative effects of β-Ecd.
study are that β-Ecd protects PC12 cells from MPP⁺-induced apoptotic cell death. Notably, the neuroprotective effects of phytoestrogen β-Ecd are most likely to be attributable to inhibition of oxidative stress, as measured by ROS, LPO, GSH/GSSG ratio, and NAD⁺/NADH ratio. Our results demonstrated for the first time evidence that antioxidant property of β-Ecd may be mediated through promoting Akt-dependent activation of Nrf2 pathway, revealed by using pharmacological inhibitors LY294002 and RNAi-based Nrf2 knockdown, respectively. Moreover, β-Ecd failed to modulate the NF-κB and calpain activity demonstrating some specificity for its action on the PI3K-Nrf2-regulated pathway.

Because depletion of estrogen is an established risk factor for PD in postmenopausal women (Rodriguez-Perez et al., 2010), phytoestrogens may provide opportunities for novel pharmacological interventions aiming at preventing or palliating the consequences of PD (Xu et al., 2009). The data presented here underscore that phytoestrogen β-Ecd elicited neuroprotective properties associated with modulation of oxidative stress that can be beneficial for early PD. The significance of the neuroprotective observations reported herein will be substantially clearer if it is known whether β-Ecd, administered in vivo, crosses the blood–brain barrier. Because of unique sensitivity to changes in O₂ availability, the PC12 cell line is frequently used as a cellular...
model to study neuronal vulnerability to oxidative stress (reviewed by Martin and Grishanin, 2003). Nevertheless, PC12 cells used in this study share the limitations common to in vitro models, namely an inability to assess cell-to-cell interactions, architectural aspects of brain regions, and related issues of bioavailability, dose, and bioeffectiveness (Markowitz and Zhu, 2012), meaning that it is difficult to extrapolate relevant in vivo concentrations of β-Ecd from in vitro results.

The available evidence in humans, albeit limited, is highly suggestive that oxidative stress has been associated with the etiology of both sporadic and monogenic forms of PD (reviewed by Hauser and Hastings, 2013). In this study, we have shown that the presence of MPP⁺ in cell culture elicited oxidative stress, which are consistent with previous studies (Wang et al., 2011). Our in vitro studies have demonstrated that β-Ecd inhibits MPP⁺-triggered oxidative stress. The NAD⁺/NADH and GSH/GSSG ratio data did not correlate exactly with each other. For example, β-Ecd at a concentration of 10 μM caused a 48% increase in NAD⁺/NADH ratio but only a 35% raise in GSH/GSSG ratio. These observed differences may be due to the factor that NAD⁺/NADH ratio and GSH/GSSG ratio do not shared mechanisms of β-Ecd antioxidative actions, while both are accepted as indicator of oxidative stress. β-Ecd indirectly attenuated oxidative stress by inducing gene expression of HO-1 and also directly inhibited oxidative stress by scavenging free radicals. This unique mechanism explains, at least partially, its potent antioxidant capacity. González-Polo et al. (2003) showed that exposure of cerebellar granule cells to MPP⁺ results in apoptotic cell death, which is markedly attenuated by co-treatment of cerebellar granule cells with the radical scavenger vitamin E. Comparison between β-Ecd and vitamin E on the antioxidative effects merits further study.

β-Ecd anti-apoptotic effects have been reported in vitro (Zhang et al., 2014). In particular, a report by Yang et al. (2015) demonstrated neuroprotective effects of β-Ecd on PC12 cells cytotoxicity induced by β-amyloid25–35. In our studies, β-Ecd further protected PC12 cells against MPP⁺-induced apoptosis, although the magnitude of protection was rather modest. Additional experiments are necessary to clarify downstream transcription factors involved in inhibition of ROS mediated apoptosis by β-Ecd.

Several therapeutic approaches targeted to oxidative stress of PD have failed, most likely because they have targeted only one aspect such as the decline of a single antioxidant (reviewed by De Araújo et al., 2011; Snow et al., 2010). Considering the complexity of the antioxidant system, it seems reasonable to consider that the induction of endogenous protective pathways, such as the Nrf2/ARE pathway against oxidative stress, is a viable strategy for delaying the progression of injury and cell death (Tufécki et al., 2011). This study demonstrates that MPP⁺ increases oxidative stress in PC12 cells and results in a small but statistically significant increased ARE promoter activity concomitant with a significant Nrf2 nuclear localization and elevation in HO-1 gene expression. However, these results were contrary to our initial expectation. Possible explanation is an antioxidant adaptive response to oxidative stress. β-Ecd-induced Nrf2 nuclear accumulation increases HO-1 expression and GSH level, which can directly act to eliminate free radicals and oxidatively damaged molecules. The enhancement of MPP⁺-inducible Nrf2 nuclear localization by β-Ecd was accompanied by enhancement of steady-state levels of mRNA for Nrf2 with only a modest correlation pattern, suggesting that posttranscriptional effect may be involved in β-Ecd-induced Nrf2 expression. To confirm the possibility that the increase in HO-1 mRNA expression was due to Nrf2 nuclear translocation, we used transient transfection of Nrf2 shRNA in PC12 cells. Using this approach, we showed that shRNA-mediated knockdown of Nrf2 abolished β-Ecd-mediated upregulation of HO-1 mRNA as well as protein expression levels. The finding corroborates our hypothesis that β-Ecd induces HO-1 by an Nrf2-dependent mechanism. To further confirm the translocation of Nrf2 into the nucleus obtained from the above Western blot assay, additional experiment, beyond the scope of this report, will be required to determine the activation of Nrf2 using ELISA-based TransAM Nrf2 kit.

Several signaling pathways including PI3K were identified as upstream pathways that regulate Nrf2 activity (Gornini et al., 2014). This study showed that β-Ecd induces phosho-Akt, which correlate with their activation status, which, in turn, activates GSK-3β. We further evaluated the role of the PI3K signaling pathway in the β-Ecd mediated-Nrf2 nuclear translocation. Experiments in this study demonstrated that the PI3K inhibitor LY294002 dramatically, if not completely, abrogated the induction of β-Ecd on ARE trans-activating activity and HO-1 expression. It remains incompletely understood how HO-1 elevation could result in inhibition of apoptosis in PC12 cells exposed to MPP⁺. MAP kinases is a key signaling molecule implicated in the regulation of a broad array of biological responses including receptor-stimulated mitogenesis, oxidative burst, and cell survival (reviewed by Son et al., 2013). However, we failed to observe a significant effect of β-Ecd on the phosphorylation of MAP kinases in our model. In this study, we addressed the question of which survival pathways upstream of the Nrf2/ARE are involved in the β-Ecd-mediated apoptosis delay. The results suggest that the activation of the PI3K/Akt pathway is a major step for β-Ecd inhibition of apoptosis in PC12 cells exposed to MPP⁺.

In our cultures, β-Ecd activates both Akt and Nrf2 pathways and caused the upregulation of HO-1. Importantly, β-Ecd-mediated survival and HO-1 upregulation disappeared in the presence of LY294002 or Nrf2 shRNA, suggesting the involvement of both pathways in neuroprotection by β-Ecd. When the combination of PI3K and Nrf2 inhibition was used, the level of reversal on β-Ecd-mediated inhibition of oxidative stress lacks additive action. This observation suggests a seemingly linear relation of the 2 signaling molecules along the action axis. Nrf2 is indeed downstream of Akt in the signal transduction pathway as shown in experiments performed with the inhibitor of PI3K and Nrf2 shRNA. LY294002 blocked β-Ecd activation of both Akt and HO-1. Nrf2 shRNA impaired HO-1 activation without any significant modification of Akt activation. Future experiments will focus on the identification of additional signaling targets of antiapoptotic effect of β-Ecd.

A growing body of evidence indicates that calpain activity is involved in MPP⁺-induced cell death (Chera et al., 2002; Harbison et al., 2011). Calpain I is typically activated in the cell by calcium ion in the micromolar range, which can be readily achieved by the loss of intracellular calcium homeostasis (Polster et al., 2005). Our studies have found no effect of β-Ecd on calpain activity. These results did not support our initial assumption that β-Ecd might reduce the calpain activity in PC12 cell exposed to MPP⁺. Our results also showed that β-Ecd failed to modulate the expression of NF-κB in PC12 cell exposed to MPP⁺, suggesting that NF-κB are unlikely to be involved in the neuroprotective effects of β-Ecd against MPP⁺-induced neurotoxicity. These results imply that PI3K-Nrf2-regulated pathway rather than MAPK and NF-κB pathway may contribute to the neuroprotection of β-Ecd.
CONCLUSION

Results in this study support our general hypothesis and demonstrate that β-Ecd neuroprotection against MPP⁺ toxicity might mainly result from its antioxidant capability by interruption of PI3K-Nrf2-regulated pathway. Based on our observations, a simplified pathway is proposed in Figure 6 to describe the possible involvement of signaling pathways in the β-Ecd inhibition apoptosis in vitro. It bears emphasis that the underlying mechanisms are certainly more complex than what described here. Several questions remain to be addressed before definitive conclusions regarding that β-Ecd effects on inhibition of nigrostriatal neurons loss in patients with PD can be made, including: (1) whether or not neuropreventive effect of β-Ecd on MPP⁺-induced apoptosis occur in vivo, (2) does β-Ecd crossed the blood–brain barrier? and (3) How about the clinical safety of β-Ecd? In concert, these data suggest that β-Ecd is a potential candidate for further preclinical study aimed at preventing or palliating the consequences of PD.

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