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N-Propargyl Caffeate Amide (PACA) Potentiates Nerve Growth Factor (NGF)-Induced Neurite Outgrowth and Attenuates 6-Hydroxydopamine (6-OHDA)-Induced Toxicity by Activating the Nrf2/HO-1 Pathway

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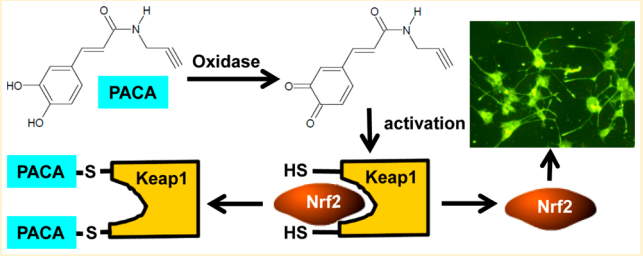
ABSTRACT: Insufficient production of neurotrophic factors is implicated in the pathogenesis of various neurodegenerative disorders. The aim of the present study was to evaluate the potential of *N*-propargyl caffeate amide (PACA) to enhance nerve growth factor (NGF)-induced neurite outgrowth and the underlying mechanisms. We discovered that PACA not only potentiated NGF-induced neurite outgrowth but also attenuated 6-hydroxydopamine (6-OHDA) neurotoxicity in dopaminergic PC12 cells and primary rat midbrain neurons. To identify the PACA-binding proteins, we introduced a biotin tag to the covalent PACA−protein adducts via "click chemistry" alkyne−azido cycloaddition. As a result, kelch-like ECH-associated protein 1 (Keap1) was isolated as the predominant protein from PACA treated PC12 cells. We demonstrated that the formation of PACA−Keap1 conjugates induced the nuclear translocation of transcription factor Nrf2 and the expression of antioxidant heme oxygenase-1 (HO-1). Importantly, specific HO-1 inhibitor SnPP diminished the neuroprotective and neuritogenic activities of PACA. Moreover, PACA attenuated 6-OHDA-induced production of neurotoxic reactive oxygen species and reactive nitrogen species. PACA also preserved mitochondrial membrane integrity and enhanced the cellular resistance against 6-OHDA neurotoxicity. These results suggest that PACA may exhibit neuroprotective and neuritogenic activities via activating the Nrf2/HO-1 antioxidant pathway.



KEYWORDS: *N-Propargyl caffeate amide (PACA), Nrf2/HO-1, neuroprotection, neuritogenic, Parkinson's disease*

Parkinson's disease (PD) is the second most common neurodegenerative disease and affects 1−2% of elderly people above the age of 65 worldwide.[1] L-DOPA, dopamine agonists, MAO-B inhibitors, and NMDA receptor antagonists have been recently evaluated to ameliorate the motor symptoms in PD patients.[2,3] None of the current therapies is clinically proven for halting progression of neurodegeneration.[4] On the other hand, neurogenesis is often impaired in aging brains, PD, and other neurodegenerative disorders due to the insufficient production of neurotrophic factors.[5] Interestingly, neurogenesis is also reduced in 6-hydroxydopamine (6-OHDA)-induced PD animal models and transgenic mice that overproduce human α-synuclein.[6] In fact, neurotrophic factor therapies have been evaluated to enhance neurogenesis for the treatment of PD and Alzheimer's disease.[7] However, the clinical applications of NGF and several other neurotrophic factors have been dampened by the delivery issues and side effects.[8] Thus, enormous effort has been made to search for small molecules to mimic or enhance the pharmacological effects of neurotrophic factors on neuroprotection and neuroregeneration.

Oxidative stress is well-known to disrupt the survival, proliferation, and differentiation of neurons in PD.[9] Nuclear factor erythroid 2-related factor 2 (Nrf2) is an important endogenous redox-sensitive transcription factor.[10,11] Under normal conditions, Nrf2 forms a protein complex with kelch-like ECH-associated protein 1 (Keap 1) in the cytosol. Under oxidative stress, Keap1 is often oxidized, thereby releasing Nrf2. Alternatively, various electrophilic inducers also disassociate the Nrf2−Keap1 complex by covalently modifying Keap1.[12,13] Consequently, Nrf2 is translocated into the cell nucleus and activates the expression of various phase II defense enzymes, antioxidant proteins, and anti-inflammatory factors.[10,11] As an example, heme oxygenase-1 (HO-1) is induced by Nrf2-mediated mechanism. HO-1 catalyzes the degradation of pro-oxidant heme to biliverdin/bilirubin, carbon monoxide, and ferrous ion. HO-1 thereby exhibits a broad range of biological activities such as antioxidant, anti-inflammatory, neuroprotec-

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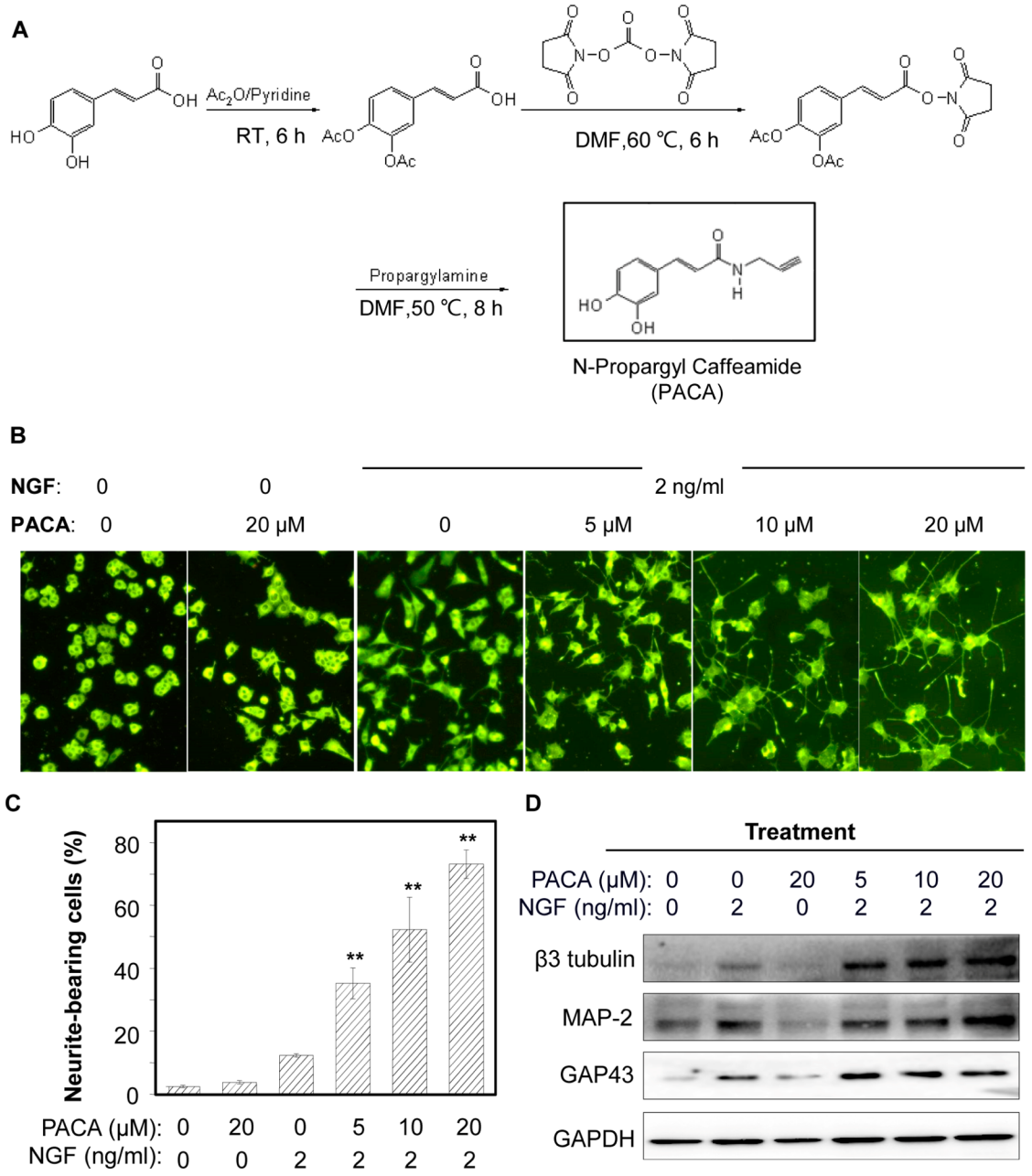

Figure 1. Synthesis and neuritogenic activity of *N*-propargyl caffeate amide (PACA). (A) Scheme illustrating the chemical synthesis of *N*-propargyl caffeamide (PACA). Ac_2O, acetic anhydride; DMF, *N*,*N*-dimethylformamide. (B) Representative images for the potentiation of NGF-induced neurite outgrowth in PC12 cells by PACA. PC12 cells were treated with NGF (2 ng/mL) and PACA, alone or in combination. The cells were stained with neurite outgrowth staining kit and imaged under a fluorescence microscope. (C) Quantification of neurite outgrowth in panel B. Following the staining of neurites, the cells bearing neurites longer than 20 μm were counted under a fluorescence microscope. **, $p < 0.01$ (sample vs NGF alone). (D) Western blot analysis of neuronal biomarkers. The cells were treated with NGF and PACA, alone or in combination, whereas the control cells were treated with vehicle for 72 h. The cellular proteins were extracted and detected by Western blot analysis for the expression of neuron markers. GAPDH was detected as the control of protein loading.

tive, and immunomodulatory activities.[14,15] Importantly, the Nrf2/HO-1 pathway also plays a key role in neurogenesis and neurite outgrowth.[16,17] Therefore, the Nrf2/HO-1 pathway becomes a therapeutic target in the treatment of various neurodegenerative disorders including PD.[12,15,18]

Natural products constitute a rich resource for the identification of neuroprotectants and neuroregenerative reagents.[19,20] Caffeic acid derivatives exert antioxidant, anti-inflammatory, chemopreventive, anticancer, and antibacterial properties in a structure-dependent and cell-type-specific manner.[21,22] We have recently demonstrated that bornyl caffeate induced apoptosis in several cancer cell lines via stimulating GSH depletion, ROS formation, and mitochondrial dysfunction.[23,24] Nevertheless, recent studies also demonstrated that caffeic acid derivatives induced brain-derived neurotrophic factor (BDNF) expression and scavenged neurotoxic peroxynitrite.[25,26] However, caffeic acid derivatives have not been investigated for neuroprotection and neuritogenesis against 6-OHDA induced neurotoxicity.

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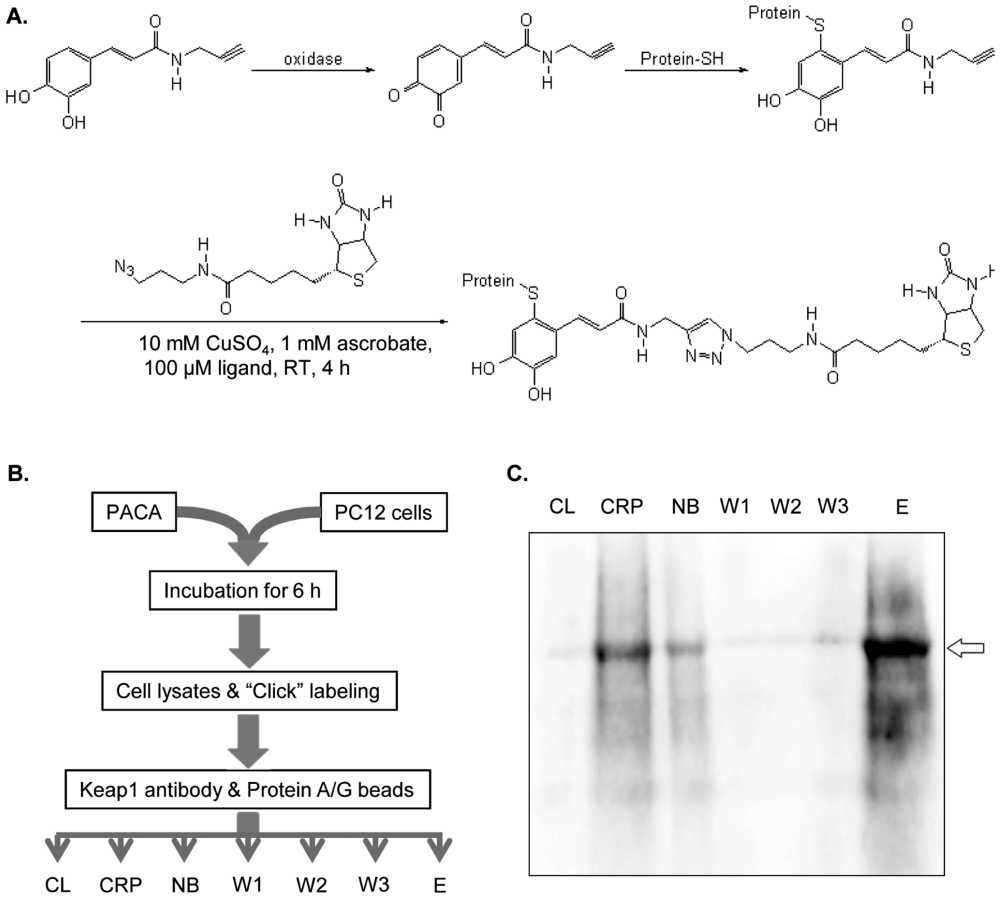

Figure 2. Identification of PACA-modified proteins. (A) Proposed mechanism for biotin labeling of PACA-modified proteins by click chemistry. Oxidase represents the enzymes that oxidize PACA to o-quinone; protein-SH represents the protein bearing free cysteine residues. (B) Procedure for the identification of PACA-modified proteins. (C) Western blotting analysis of PACA-modified proteins. PC12 cells were treated with PACA (20 μM) for 6 h. The cellular proteins were isolated and treated with azido-biotin under click chemistry conditions. Click chemistry products were incubated with Keap1 antibody and subsequently isolated by Protein A/G agarose beads. All protein fractions were resolved by SDS-PAGE and detected by streptavidin–HRP conjugate. CL, cell lysates of PACA-treated PC12 cells; CRP, click chemistry products; NB, nonbound fraction; W1, washing fraction-1 with PBS; W2, washing fraction-2 with PBS; W3, washing fraction with PBS; E, elution by heating the beads in 2× Laemmli sample buffer at 95 °C for 5 min.

In the present study, we initially synthesized a new *N*-propargyl caffeate amide (PACA) as a probe for the identification of the PACA–protein adducts in rat dopaminergic PC12 cells. By introducing a biotin tag into the PACA–protein adducts through the well-established "click" alkyne–azido cycloaddition approach,[27] we identified the PACA-modified proteins by a proteomic approach and investigated the molecular mechanisms underlying the neuroprotective and neuritogenic activities of PACA.

RESULTS

Synthesis and Chemical Characterization of PACA. PACA bearing an alkyne group was chemically synthesized through a three-step procedure as illustrated in Figure 1A. Caffeic acid was first acetylated with acetic anhydride in pyridine to yield di-O-Ac-caffeic acid. Di-O-Ac-caffeic acid was subsequently converted into di-O-Ac-caffeic acid *N*-hydroxysuccinimide ester via the reaction with *N*,*N*′-disuccinimidyl carbonate in DMF. Without purification, the crude di-O-Ac-caffeic acid *N*-hydroxysuccinimide ester formed an amide product with propargylamine. The O-acetyl groups were simultaneously removed by propargylamine at room temperature, while the deacetylation was promoted with the increase of temperature to 50 °C. PACA as the final product was purified by silica gel chromatography and characterized by MS and NMR. PACA, light yellow power, HRESI-MS $[M - H]^-$ (m/z), calcd for $C_{12}H_{10}NO_3$ 216.0666, found 216.0665. ESI-MS (m/z): 218 $[M + H]^+$, 435 $[2M + H]^+$. ^{1}H NMR (MeOD/$CDCl_3$, 400 MHz), δ_H: 2.79 (1H, t, 2.4 Hz, H-3″), 4.50 (2H, d, 2.4 Hz, H_2-1″), 6.74 (1H, d, 15 Hz, H-2), 7.21 (1H, d, 8.1 Hz, H-6′), 7.33 (1H, d, 8.1 Hz, H-5′), 7.48 (1H, s, H-2′), 7.86 (1H, d, 15 Hz, H-3), 8.33 (1H, s, H–N). ^{13}C NMR (MeOD/$CDCl_3$, 75 MHz), δ_C: 28.5 (C-3″), 70.9 (C-1″), 79.11 (C-2″), 113.7 (C-2′), 115.1 (C-5′), 116. 4 (C-2), 121.3 (C-6′), 126.6 (C-1′), 141.5 (C-3), 144.5(C-3′), 146.7 (C-4′), 167.3 (C-1).

PACA Potentiated NGF-Induced Neuritogenesis. To evaluate the neuritogenic activity of PACA, we treated PC12 cells with NGF (2 ng/mL) alone or in combination with PACA at the concentrations of 5, 10, or 20 μM for 72 h. The cells were stained by a neurite outgrowth kit and subsequently analyzed for neurites (>20 μm) under a fluorescence microscope. Compared with NGF alone as shown in Figure 1B,C, PACA significantly potentiated NGF-induced neurite outgrowth in a concentration-dependent manner. PACA (20 μM) alone did not induce neurite outgrowth. Interestingly, PACA (20 μM) and NGF (2 ng/mL) in combination increased

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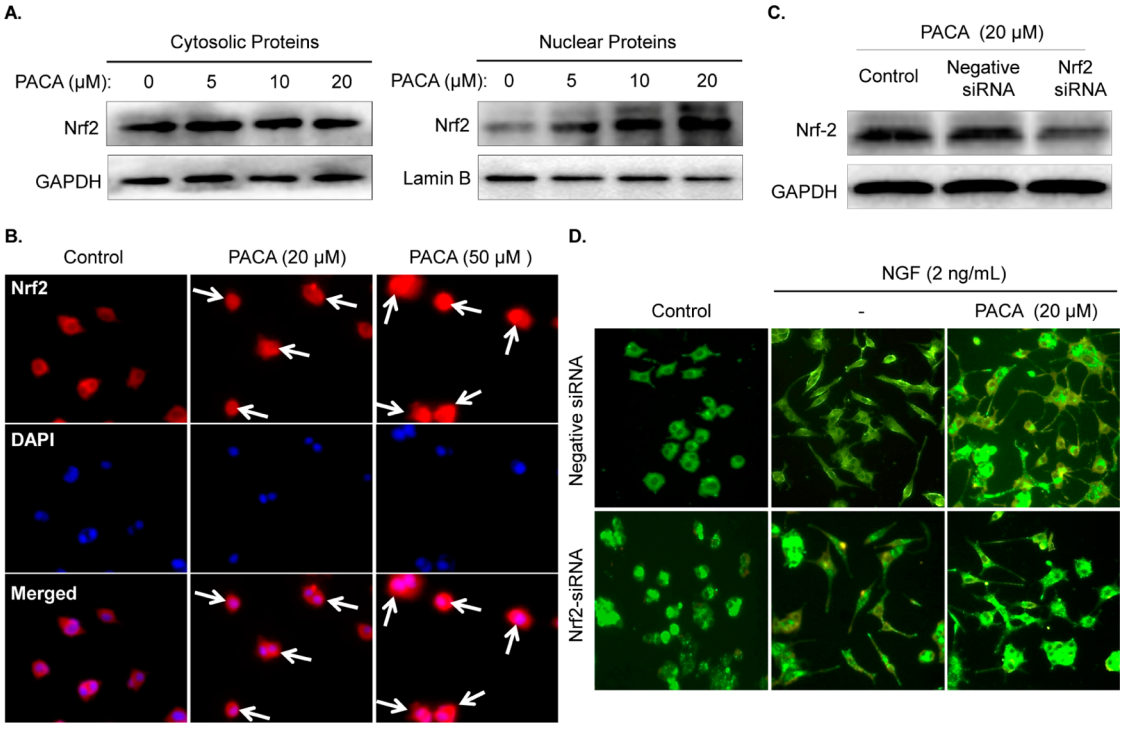

Figure 3. PACA induced the nuclear translocation of Nrf2 and subsequent neurite outgrowth. (A) Western blotting analysis of the cytosolic and nuclear Nrf2. Following PACA treatment, the cytosolic and nuclear proteins were isolated and analyzed by Western blotting for Nrf2 expression. GAPDH and lamin b were analyzed as the control of protein loading. (B) Immunofluorescence staining of Nrf2. Following PACA treatment, the cells were probed with Nrf2 antibody and subsequently visualized with Alexa Fluor 586-conjugated secondary antibody. DAPI was used to detect the cell nuclei. The images were captured under a fluorescence microscope. The arrows point to the nuclear Nrf2. (C) siRNA-mediated silencing of Nrf2 expression. After the transfection with Nrf2 siRNA or negative control siRNA for 48 h, PC12 cells were analyzed for Nrf2 expression. (D) Correlation between the level of Nrf2 expression and the induction of neurite outgrowth by PACA and NGF. Following siRNA transfection, PC12 cells were treated with NGF and PACA, alone or in combination, for 72 h. The cells were stained with neurite outgrowth staining kit and imaged on a fluorescence microscope. Representative images were shown.

the number of neurite-bearing cells by approximately 6-fold relative to NGF alone. Following the treatment with PACA and NGF, alone or in combination, PC12 cells were analyzed by Western blotting for the expression of neuronal biomarkers (i.e., β3-tubulin, MAP2, and GAP-43). As shown in Figure 1D, PACA clearly potentiated the activity of NGF in the induction of neuronal markers including β3-tubulin, MAP2, and GAP-43. Collectively, these results suggested that PACA could potentiate the neuritogenic activity of NGF in the extension of neurites.

Identification of Keap1 as the Predominant PACA-Modified Protein. To identify the PACA-modified proteins, we introduced a biotin tag to PACA-modified proteins for versatile affinity purification through a "click" alkyne−azido cycloaddition (Figure 2A). In our pilot study, the cellular proteins were prepared from PACA-treated PC12 cells, incubated with azido-biotin under "click" chemistry reaction conditions.[27] Biotinylated proteins were initially isolated by streptavidin-agarose beads from Sigma-Aldrich (St Louis, MO, USA). Following gel electrophoresis and Western blotting onto PVDF (polyvinylidene difluoride) membranes, HRP−streptavidin conjugate detected a predominant ~70 kDa protein band (data not shown). Such protein band was also detected by Keap1 antibody through a similar procedure.

To directly isolate PACA-modified Keap1 from the cell culture system, biotinylated proteins were pulled down by Keap1 antibody and Protein A/G beads as outlined in Figure 2B. All protein fractions were resolved by gel electrophoresis and Western blotting onto PVDF membranes. The blots were probed by HRP-conjugated streptavidin and subsequently detected by chemiluminescence. As shown in Figure 2C, the 70 kDa protein band was predominantly captured by Keap1 antibody.

PACA Induced the Translocation of Nrf2 into Cell Nucleus. To verify the potential role of PACA in the activation of the Keap1−Nrf2 pathway, we examined the intracellular localization of Nrf2 by Western blotting and immunostaining methods. After PACA treatment, the cellular proteins were separated in the cytosolic and nuclear fractions and analyzed by Western blotting with Nrf2 antibodies. GAPDH was used as the control of protein loading, whereas lamin B was detected as the nuclear biomarker. As shown in Figure 3A, PACA significantly increased the levels of nuclear Nrf2 in a concentration-dependent manner. Based on immunostaining of the intracellular Nrf2, on the other hand, PACA also promoted the accumulation of Nrf2 in the cell nucleus (Figure 3B).

To explore the role of Keap1-mediated Nrf2 activation in the neuritogenic activity of PACA, we attempted to knock-down the expression of Nrf2 by specific siRNA. As shown in Figure 3C, compared with negative control siRNA, Nrf2-siRNA markedly decreased the expression level of Nrf2 protein. Importantly, PACA was no longer able to potentiate NGF-induced neurite outgrowth in Nrf2 siRNA-transfected PC12 cells, whereas negative control siRNA did not affect the neuritogenic activity of PACA (Figure 3D).

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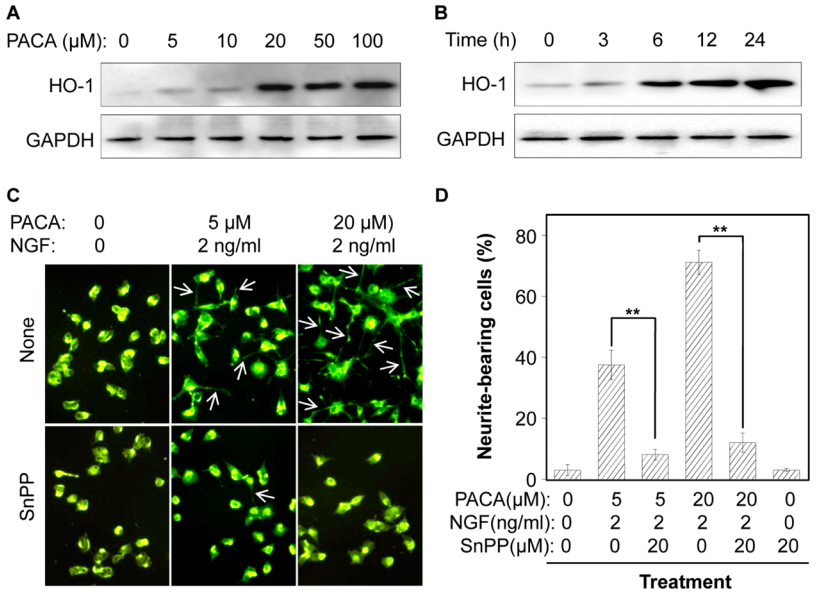

Figure 4. Potential role of HO-1 induction in the effect of PACA on NGF-induced neurite outgrowth. (A, B) Western blot analysis of HO-1 induction. Following PACA treatment at different concentrations (A) and for different time points (B), the cellular proteins were isolated and analyzed by Western blotting for HO-1 expression. GAPDH was analyzed as the control of protein loading. (C) Effect of HO-1 specific inhibitor on the neuritogenic activity of PACA. PC12 cells were treated with NGF (2 ng/mL) in combination with PACA (5 or 20 μM) in the presence or absence of HO-1 inhibitor SnPP for 72 h. Cells were stained by neurite outgrowth staining kit and imaged under a fluorescence microscope. (D) HO-1 dependence on the effect of PACA on NGF-induced neurite outgrowth. The images captured in panel C were analyzed for the number of neurite bearing cells. The cells bearing neurites longer than 20 μm were counted. The values represent means ± SD (n = 3). **, $p < 0.01$ (+SnPP vs −SnPP).

HO-1 Induction Plays a Key Role in the Neuritogenic Activity of PACA. To further examine the effects of PACA on Nrf2 target genes, we focused on the induction of HO-1 expression in response to PACA treatment. We analyzed the cellular proteins from PC12 cells following the treatment with PACA at concentrations ranging from 5 to 100 μM for 24 h or at the concentration of 20 μM for 3, 6, 12, or 24 h. Based on Western blotting results (Figure 4A,B), PACA indeed induced HO-1 expression in a time- and concentration-dependent manner.

To clarify the neuritogenic role of HO-1 induction, we treated PC12 cells with NGF, PACA, and HO-1 inhibitor SnPP, alone or in combination, for 72 h. The neurites were visualized by neurite outgrowth staining kit and imaged under a fluorescence microscope (Figure 4C). By using NIH ImageJ software, we compared the number of neurite-bearing cells in each treatment. As shown in Figure 4D, HO-1 inhibitor SnPP significantly diminished the potential of PACA in the potentiation of NGF-induced neurite outgrowth.

PACA Attenuated 6-OHDA-Induced Superoxide Ion Generation, NO Production, and Mitochondrial Membrane Depolarization. To investigate the neuroprotective mechanisms of PACA, we treated PC12 cells with 6-OHDA alone or in combination with PACA at concentrations of 5, 10, 20, and 50 μM. At the end of treatment, we determined the intracellular levels of superoxide ions and NO by specific probes, DHE and DAF-FM DA, respectively. We found that PACA significantly attenuated 6-OHDA-induced formation of not only superoxide ions (Figure 5A,B) but also neurotoxic NO (Figure 5C,D) in a concentration-dependent manner. We further examined the protection by PACA of mitochondrial membrane integrity by using a cell permeable fluorescent probe JC-1. As shown in Figure 5E, 6-OHDA fatally disrupted the mitochondrial membrane integrity as indicated by the leakage of JC-1 into the cytosol. Interestingly, PACA maintained MMP against 6-OHDA-induced membrane disruption and subsequent leakage of probe JC-1. Our results suggested that PACA maintained the mitochondrial membrane integrity in a concentration-dependent manner.

Role of HO-1 Induction in the Neuroprotection by PACA. To examine the effect of PACA on the survival and growth against 6-OHDA neurotoxicity, we treated PC12 cells with 6-OHDA alone or in combination with PACA at the concentrations of 5, 10, 20, and 50 μM for 24 h. The cell viability was determined by standard MTT assay. As shown in Figure 6A, PACA enhanced the cellular resistance against 6-OHDA toxicity in a concentration-dependent manner. We further assessed the role of HO-1 induction in the cellular defense against 6-OHDA toxicity. Based on the cell viability after drug treatment, HO-1 inhibitor SnPP significantly antagonized the protective effect of PACA against 6-OHDA toxicity (Figure 6B).

To verify the neuroprotective effect of PACA, we isolated primary midbrain neurons from rat embryos. After being cultured for 6 days, primary neurons were treated with 6-OHDA alone or in combination with PACA at concentrations of 5, 10, 20, and 50 μM for 24 h. The cell viability was determined by standard MTT assay. As shown in Figure 6C, PACA increased the cell viability of primary neurons against 6-OHDA neurotoxicity in a concentration-dependent manner. Moreover, the apoptotic neurons were detected by staining with PI, whereas the cell monolayers were stained with Hoechst 33342. As shown in Figure 6D,E, PACA reduced the number of apoptotic neurons due to 6-OHDA neurotoxicity in a concentration-dependent manner.

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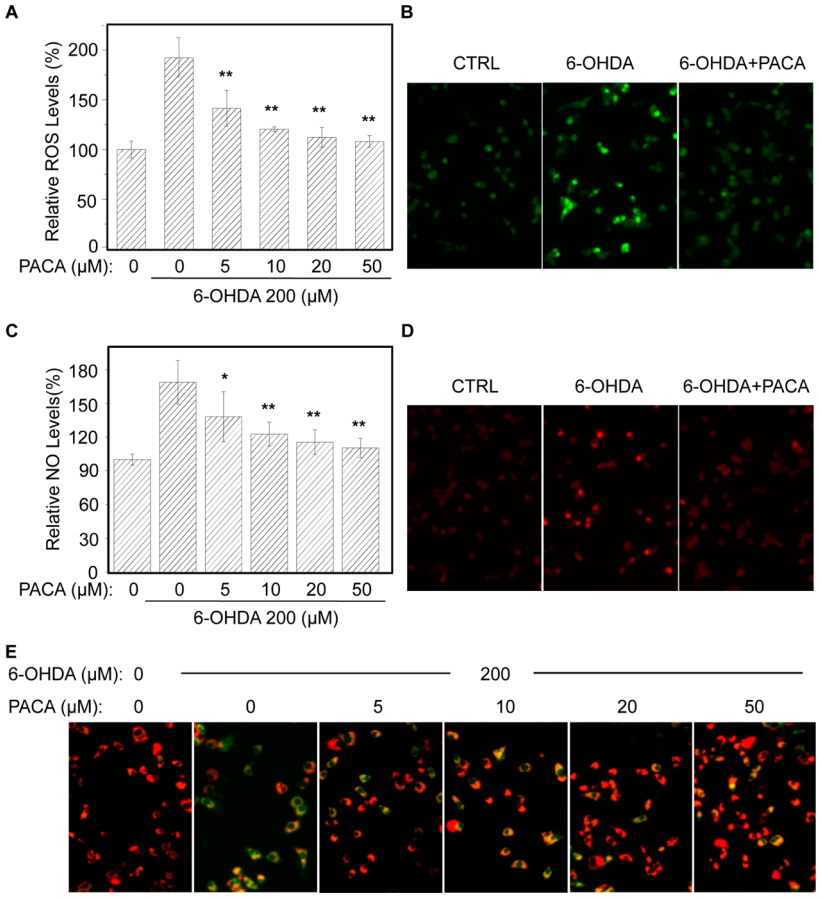

Figure 5. PACA antagonized the effects of 6-OHDA on ROS formation, NO production, and mitochondrial membrane potential. (A) PACA inhibited 6-OHDA-induced formation of superoxide ions. PC12 cells were treated with 6-OHDA alone or in combination with PACA for 6 h and subsequently stained with a probe DHE. The images were captured under a fluorescence microscope. The fluorescence was determined by ImageJ software. (B) Representative images of DHE-stained cells. 6-OHDA, 200 μM; PACA, 50 μM. (C) PACA inhibited 6-OHDA-induced NO production. PC12 cells were treated with 6-OHDA alone or in combination with PACA for 6 h and subsequently stained with a probe DAF-FM DA. The images were captured under a fluorescence microscope. The fluorescence was determined by ImageJ software. (D) Representative images of DAF-FM DA-stained cells. 6-OHDA, 200 μM; PACA, 50 μM. (E) PACA recovered mitochondrial membrane potential against 6-OHDA toxicity. PC12 cells were treated with 6-OHDA alone or in combination with PACA for 6 h and subsequently stained with JC-1. The images were captured under a fluorescence microscope.

DISCUSSION

Natural products constitute an important resource for the discovery of drug candidates against neuronal injury and neurodegeneration.[19,20] We have recently identified several botanical drugs including amygdalin and puerarin for the promotion of neuronal survival, growth, and regeneration.[28−30] Caffeic acid derivatives are also known to protect neurons from oxidative injury.[31,32] However, caffeic acid derivatives have not been fully explored for the potential to promote neuronal regeneration. In our pilot study, we synthesized a new caffeic acid derivative, PACA, and discovered that PACA effectively potentiated NGF-induced neurite outgrowth. Thus, the aim of the present study was to characterize the molecular mechanisms underlying the neuroprotective and neuritogenic activities of PACA. By analyzing the cellular covalent PACA−protein adducts, we discovered Keap1 as the predominant PACA-modified protein. We further investigated the mechanistic impact of Keap1 modification in the neuroprotective and neuritogenic activities of PACA.

Previous studies showed that Nrf2 activation ameliorated PD and PD-like behavioral symptoms, whereas mice lacking Nrf2 expression were vulnerable to neurotoxin-induced injury.[18,33] Recent *in vitro* studies showed that Nrf2 overexpression promotes neuronal differentiation in neural stem/progenitor and SH-SY5Y cells.[16,17] Thus, current research interest has been focusing on the pharmacological dissociation of the Nrf2−Keap1 complex.[18,33,34] It is well-known that natural catechol is readily converted to o-quinones by the intracellular oxidases, rendering the chemical structure reactive toward the cysteine residues of Keap1 and subsequent activation of Nrf2.[12,35] The formation of PACA−-protein adducts may not be reversible inside the cells. Thus, upon covalent modification by PACA, Keap1 becomes permanently inactivated and is no longer able to sequester Nrf2 in the cytosol. Consequently, Nrf2 is translocated to the cell nuclei and induces the expression of various antioxidant genes such as HO-1. In the present study, we also tested the chemical reactivity of PACA toward Keap1. One of the key findings was the application of "click" alkyne−azido cycloaddition to conjugate the alkyne group in PACA with an azido group in the biotin derivative.[27] We thereby isolated PACA-modified proteins by streptavidin affinity purification and detected a predominant ∼70 kDa protein band by Western blotting with HRP−streptavidin conjugate. We subsequently observed that the Keap1 antibody also

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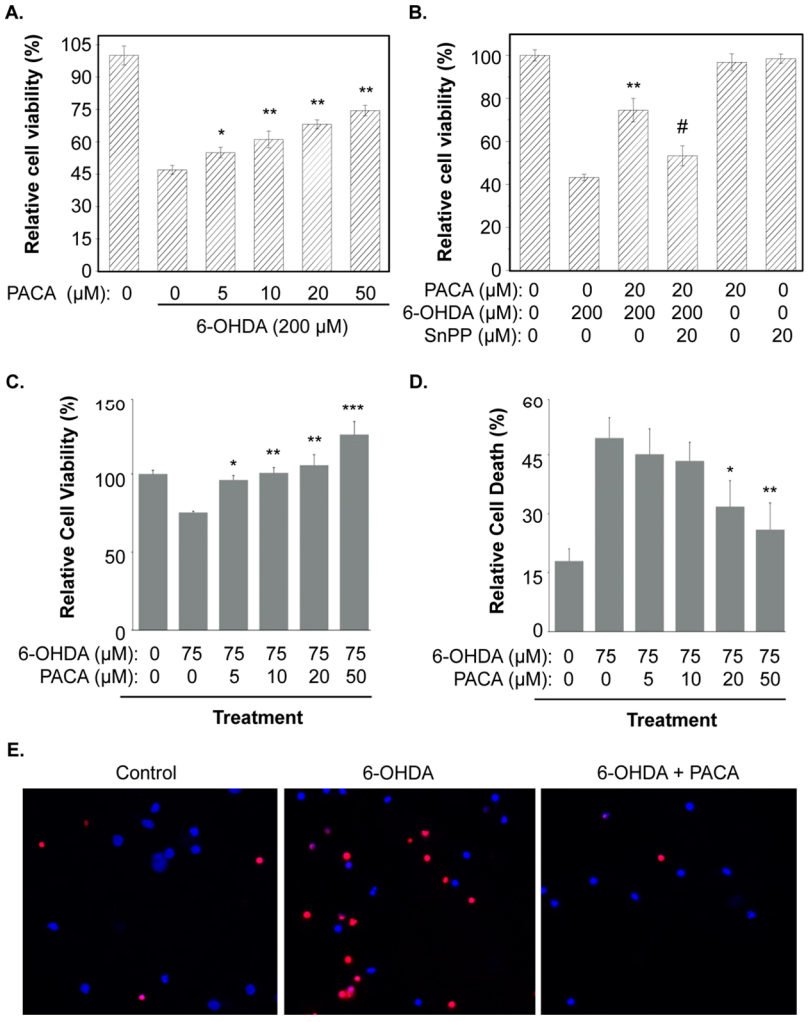

Figure 6. PACA enhanced the neuronal survival against 6-OHDA neurotoxicity via a HO-1 dependent mechanism. (A) Concentration dependent cytoprotection of PACA against 6-OHDA toxicity. PC12 cells were treated with 6-OHDA alone or in combination with PACA for 24 h. The cell viability was examined by standard MTT assay. The values represent means ± SD (n = 3). *, $p < 0.05$; **, $p < 0.01$ (6-OHDA + PACA vs 6-OHDA alone). (B) Potential role of HO-1 induction in the cytoprotection of PACA. Cells were treated with 6-OHDA alone or in combination with PACA in the presence or absence of SnPP for 24 h. The cell viability was determined by standard MTT assay. The values represent means ± SD (n = 3). **, $p < 0.01$ (6-OHDA + PACA vs 6-OHDA alone). #, $p < 0.05$ (6-OHDA + PACA + SnPP vs 6-OHDA + PACA). (C) Concentration dependent enhancement of neuronal survival against 6-OHDA neurotoxicity by PACA. Primary rat embryonic midbrain neurons were treated with 6-OHDA alone or in combination with PACA for 24 h. The neuronal viability was examined by standard MTT assay. The values represent means ± SD (n = 3). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ (6-OHDA + PACA vs 6-OHDA alone). (D) Concentration dependent reduction of 6-OHDA neurotoxicity by PACA. Primary rat embryonic midbrain neurons were treated with 6-OHDA alone or in combination with PACA for 24 h. The neurons were incubated with PI/Hoechst 33342. PI-positive and Hoechst 33342-stained neurons were counted under a fluorescence microscope. The values represent means ± SD (n = 3). *, $p < 0.05$; **, $p < 0.01$ (6-OHDA + PACA vs 6-OHDA alone). (E) Representative images for the neuroprotection of PACA against 6-OHDA neurotoxicity. 6-OHDA, 75 μM; PACA, 50 μM.

detected the predominant PACA-modified protein in a highly similar fashion. Thus, the present study succeeded in the affinity isolation of the cellular PACA−Keap1 adducts by integrating the immunoprecipitation with Keap1 antibody and subsequent Western blot detection with HRP−streptavidin conjugate. Importantly, we also confirmed that PACA induced Nrf2 nuclear translocation and subsequent HO-1 expression.

We further investigated the neuroprotective and neuritogenic effects of PACA in rat dopaminergic PC12 as an *in vitro* cell model.[36,37] In our pilot experiments, PACA alone did not show any activity to promote neurite outgrowth. However, we first discovered that PACA significantly potentiated NGF-induced neurite outgrowth when NGF was provided at low concentration (2 ng/mL). Importantly, our results revealed that PACA exhibited neuritogenic activity in a Nrf2- and HO-1 dependent manner. Following the treatment with PACA and NGF, the cells well expressed neuronal biomarkers including MAP2, β-3 tubulin, and GAP43 based on our Western blot analysis. Second, PACA significantly attenuated 6-OHDA-induced overproduction of superoxide ions and NO. Both superoxide ions and NO are hallmarks of cellular oxidative stress, leading to the disruption of MMP and apoptosis. PACA indeed suppressed the loss of MMP in 6-OHDA-treated cells as indicated by JC-1 staining. Finally, we found that PACA enhanced the cell viability against 6-OHDA toxicity in a HO-1-dependent manner. We verified that PACA enhanced the neuronal survival and reduced neuronal death against 6-OHDA neurotoxicity in primary rat midbrain neuron culture.

In conclusion, the present study described a simple synthetic procedure for the preparation of PACA as a potent neuro-

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protective and neuritogenic drug candidate against the insufficiency of NGF. We successfully identified the predominant formation of PACA−Keap1 adducts and verified that PACA induced Nrf2 activation and subsequent HO-1 expression. Importantly, the activation of the Nrf2/HO-1 pathway may mediate the neuroprotective and neuritogenic effects of PACA as outlined in Figure 7. Thus, these results may pave the avenue for future development of PACA-based drugs for treatment of PD and related neurodegenerative diseases.

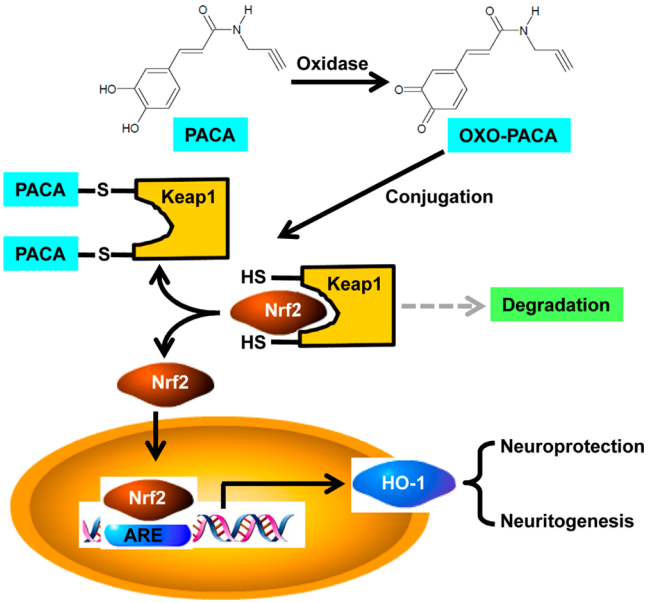

Figure 7. Diagram summarizing the actions of PACA on the Keap1/Nrf2 pathway and subsequent HO-1 induction. PACA covalently modifies Keap1, releasing Nrf2 from physiological proteolytic degradation. Following the translocation into cell nucleus, Nrf2 activates the expression of HO-1. HO-1 mediates the neuroprotective and neuritogenic activities of PACA.

METHODS

Antibodies and Biochemical Reagents. Antibodies against microtubule-associated protein 2 (MAP2), β3-tubulin, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and Alexa Fluor 594-conjugated goat anti-rabbit IgG antibodies were purchased from Cell Signaling Technology (Boston, MA, USA). HO-1 antibody was purchased from Stress gene (Ann Arbor, MI, USA). Antibodies against Nrf2, GAP43, and lamin B and horseradish peroxidase (HRP)-conjugated anti-rabbit IgG secondary antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). HRP−streptavidin conjugate and HRP-conjugated anti-rabbit IgG secondary antibody were purchased from Sigma-Aldrich (St. Louis, MO, USA). Nerve growth factor (NGF), collagen I, and other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless indicated otherwise.

Synthesis and Chemical Characterization of PACA. Caffeic acid (0.5 g) was acetylated by acetic anhydride (1 mL) in anhydrous pyridine (2 mL) at room temperature overnight to yield di-O-Ac-caffeic acid (579 mg, ∼90%). Di-O-Ac-caffeic acid was converted into di-O-Ac-caffeic acid N-hydroxysuccinimide ester via reaction with *N*,*N*′-disuccinimidyl carbonate (1.28 g) in *N*,*N*-dimethylformamide (2 mL). Following the removal of solvents by rotatory evaporation under reduced pressure, the residues containing di-O-Ac-caffeic acid *N*-hydroxysuccinimide ester were treated with propargylamine (320 μL, 275 mg) at 50 °C for 8 h. After the reaction was completed according to TLC detection, the reaction mixture was purified by gel chromatography on a silica column, yielding PACA (325 mg, 60%). PACA was further characterized by mass spectroscopy (MS) and nuclear magnetic resonance (NMR) spectroscopy. NMR spectra were recorded in MeOD/$CDCl_3$ (50:50, v/v) on a Varian Unity plus NMR 400 MHz spectrometer (Varian Inc., Palo Alto, CA, USA). MS analysis was performed on an ABI/Sciex triple quadrupole 3200 QTRAP mass spectrometer (Framingham, MA, USA) equipped with a TurboV Source operating in positive ionization mode under the control of Analyst v1.4.2 data system (Applied Biosystems/MDS Sciex, Concord, ON, Canada).

Cell Culture. Rat pheochromocytoma PC12 cell line was purchased from the American Type Culture Collection (Manassas, VA, USA), and maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% horse serum (HS) and 5% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA) at 37 °C in a humidified 5% CO_2 atmosphere.

Primary Rat Embryonic Midbrain Neurons. Primary rat midbrain neurons were isolated from 17-day-old Sprague−Dawley (SD) rat embryos as described.[38] Briefly, the midbrain neurons were carefully dissociated and seeded onto 6-well plates at a density of 1×10^6 cells/well and cultured in NeuroBasal medium containing 2% B27 supplement (Thermo Fisher Scientific Inc., Waltham, MA, USA) for 5−7 days prior to drug treatment.

Isolation and Identification of Covalent PACA−Protein Adducts. PC12 cells (1×10^7) were treated with 50 μM PACA for 6 h and subsequently lysed in 0.5 mL of 10 mM Tris-HCl buffer (pH 7.0) containing 2% SDS, 150 mM NaCl, and 1 mM DTT on ice for 30 min. The cellular proteins were denatured by heating at 95 °C for 5 min and recovered by centrifugation at 13000 rpm for 10 min to remove insoluble materials. Free thiols were blocked with 100 mM iodoacetate amide at room temperature for 2 h. After three extractions with 1 mL of butanol, the aqueous phase was recovered. The protein mixture was treated with 100 μM azido-biotin in a click chemistry reaction mix containing 1 mM sodium ascorbate, 100 μM tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine, 1 mM $CuSO_4$) at RT for 4 h. The reaction mixture was dialyzed 6 times through 10 kDa cutoff membranes against 1 mM Tris-HCl buffer (pH 7.0) containing 15 mM NaCl. The sample was incubated with 4 μg of Keap1 antibody at 4 °C overnight on roller shaker and subsequently mixed well with 200 μL of Protein A/G beads from Santa Cruz Biotechnologies (Santa Cruz, CA, USA). After the incubation at 4 °C for 4 h, the mixture was subjected to centrifugation at 2500 rpm at 4 °C for 5 min. The supernatant was carefully collected as nonbound (NB) fraction. The pellet was washed 3 times with 1 mL of PBS, yielding fractions wash-1 (W1), wash-2 (W2), and wash-3 (W3) by similar centrifugation. The bound proteins were denatured by heating at 95 °C for 5 min and separated by centrifugation at 13000 rpm for 10 min. The supernatant was carefully recovered as elution (E). Finally, all protein fractions were concentrated using EMD Millipore microcon centrifugal filter devices (10 kDa cutoff) from Billerica, MA, USA. The proteins were resolved in a 10% SDS-PAGE gel and detected with HRP−streptavidin conjugate. The bound HRP−streptavidin conjugate was visualized by Amersham ECL Select Western blotting detection reagent from GE Healthcare Biosciences (Uppsala, Sweden).

siRNA-Mediated Silencing of Nrf2 Expression. To transiently knock-down Nrf2 expression, PC12 cells were grown on poly(L-lysine) precoated coverslips for 24 h and then transfected with Nrf2 siRNA or negative control siRNA, respectively, using HiPerFect transfection reagent according to the manufacturer's instructions. After 24 h post-transfection incubation, the cells were divided into two portions. One portion of the transfected cells was subjected to Western blot analysis for Nrf2 expression. The other portion of the cells was treated with PACA and NGF, alone or in combination, for 72 h. At the end of drug treatment, the cells were stained by the neurite outgrowth staining kit according to the manufacturer's instructions. The images were captured under a Zeiss fluorescence microscope (Carl Zeiss, Germany). The average neurite length for individual differentiated cells was analyzed by ImageJ software (http://rsbweb.nih.gov/ij/).

Assay of Neurite Outgrowth. Neurite outgrowth was measured by neurite outgrowth staining kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Briefly, PC12 cells were treated with PACA and NGF, alone or in combination, for indicated

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time. The neurites were stained with neurite outgrowth staining kit for 20 min at 37 °C. Following two washes with phosphate-buffered saline (PBS), the cells were incubated with background suppression dye. The images were acquired under a Zeiss fluorescence microscope (Carl Zeiss, Germany). The cells bearing neurites longer than 20 μm in three views were counted relative to the total cells from the cell monolayer per view. The values represent the means ± SD (n = 3).

Western Blotting Analysis. The cellular proteins were extracted from the cells after drug treatment as indicated and resolved by gel electrophoresis in 10% SDS-polyacrylamide gels. The protein bands were subsequently transferred onto PVDF membranes. Following blocking with 5% nonfat milk powder or BSA in TBS-T (Tris-buffered saline with 0.2% Tween-20) buffer overnight, the blots were probed with specific primary antibodies, detected by secondary antibody–HRP conjugate, and visualized by Amersham ECL Select Western blotting detection reagent from GE Healthcare Biosciences (Uppsala, Sweden).

Cell Culture Immunofluorescence Staining. PC12 cells were treated with PACA at the indicated concentrations for 6 h, the cells were fixed with 4% paraformaldehyde in PBS for 30 min, permeabilized in 0.5% Triton X-100 in PBS for 30 min and blocked in 5% normal goat serum in PBS at room temperature for 1 h. The cells were incubated with Nrf2 antibody. After 5 washes with 1× TBS/0.1% Tween-20 buffer, the bound antibodies were detected by Alexa Fluor 594-conjugated goat anti-rabbit IgG secondary antibody, whereas the cell nuclei were stained with DAPI for 15 min. The fluorescent images were acquired under a Zeiss fluorescence microscope (Carl Zeiss, Germany).

Measurement of Cell Viability. The cell viability was determined by a standard colorimetric assay using 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT). In brief, PC12 cells or primary neurons were seeded in a 96-well microplate for 24 h or 6 days and treated with 6-OHDA, PACA, and SnPP, alone or in combination, for a certain period of time. The cell monolayers were subsequently incubated with 0.5 mg/mL MTT in PBS for 4 h. After the solubilization of purple formazan in DMSO, the absorbance at 570 nm was measured on a microplate reader (Bio-Rad, Hercules, CA, USA). The cell viability was relatively presented as a percentage of vehicle-treated controls.

Measurement of Mitochondrial Membrane Potential (MMP). MMP was assessed with a lipophilic cationic fluorescent dye 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-benzimidazol-carbocyanine iodide (JC-1) according to the manufacturer's instruction. At the end of drug treatment, the cells were incubated with 1 μg/mL JC-1 at 37 °C for 10 min. After excitation at the wavelength of 485 nm, the fluorescence at the emission wavelengths of 530 and 590 nm was visualized under a Zeiss fluorescence microscope (Carl Zeiss, Göttingen, Germany).

Measurement of Intracellular Superoxide Ions and Nitric Oxide (NO). The intracellular levels of superoxide ions and NO were evaluated by staining with dihydroethidium (DHE) and 4-amino-5-methylamino-2′,7′-difluororescein diacetate (DAF-FM DA) as previously described.[39] Briefly, at the end of drug treatment, the cells were staining with DHE or DAF-FM DA. The images were captured under a Zeiss fluorescent microscope (Carl Zeiss, Germany). The intracellular levels of superoxide ions and NO were analyzed using ImageJ software (http://imagej.nih.gov/ij/) and presented as a percentage of vehicle controls.

Hoechst 33342/Propidium Iodide (PI) Staining. Primary rat midbrain neurons were seeded in 12-well plates and cultured at 37 °C for 6 days. The cells were treated with 75 μM of 6-hydroxydopamine (6-OHDA) with or without PACA at the concentrations of 5, 10, 25, and 50 μM for 12 h. At the end of treatment, the neurons were stained with 5 μM of Hoechst 33342 and 1 μM of PI in the differentiation medium at 37 °C for 30 min. Both Hoechst- and PI-positive cells were counted under a Zeiss fluorescence microscope (Carl Zeiss, Germany).

Statistical Analysis. The results were presented as means ± SD and analyzed by one-way analysis of variance (ANOVA) test with SPSS 13.0 software. A p-value of <0.05 was considered statistically significant.

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Author Contributions

Chuanbin Yang, Jia Zhao, and Yuanyuan Cheng performed experiments. Jianhui Rong and X Chris Le designed the study. Chuanbin Yang and Jianhui Rong wrote the manuscript.

Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

MAO B, monoamine oxidase B; NMDA, *N*-methyl-D-aspartate; Nrf2, nuclear factor erythroid-2-related factor 2; HO-1, heme oxygenase-1; PACA, *N*-propargyl caffeate amide; NGF, nerve growth factor 2; Keap1, protein kelch-like ECH-associated protein 1; PD, Parkinson's disease; CAPE, caffeic acid phenethyl ester; ROS, reactive oxygen species; RNS, reactive nitrogen species; DHE, dihydroethidium; SnPP, tin protoporphyrin IX; MMP, mitochondrial membrane potential; 6-OHDA, 6-hydroxydopamine; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-benzimidazol-carbocyanine iodide

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