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PINK1/parkin-mediated mitophagy pathway is related to neuroprotection by carnosic acid in SH-SY5Y cells

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ABSTRACT

Impairment in mitophagy contributes to the pathology of Parkinson's disease. This study investigated whether Phosphatase and tensin homologue (PTEN)-induced kinase 1 (PINK1)/parkin-mediated mitophagy is linked to the protective effect of carnosic acid (CA) from rosemary. Treatment of SH-SY5Y cells with 6-hydroxydopamine (6-OHDA) disrupted the mitochondrial membrane potential, inhibited voltage-dependent anion channel 1 (VDAC1) protein, and induced cytosolic *cytochrome c*, but CA pretreatment reversed these findings. By immunofluorescence, CA pretreatment was shown to increase the co-localization of red fluorescence (parkin) and MitoTracker green FM fluorescence (mitochondria), indicating that CA promoted the translocation of parkin into mitochondria. Immunoprecipitation with VDAC1 antibody showed that 6-OHDA treatment decreased the interaction of ubiquitinated protein with VDAC1. However, CA pretreatment reversed this reduction in the interaction of ubiquitinated protein with VDAC1. Silencing of PINK1 and parkin by use of small interfering RNA (siRNA) attenuated the ability of CA to reverse 6-OHDA-inhibited autophagic vacuoles. Moreover, in PINK1 siRNA-transfected cells, CA no longer reversed these actions of 6-OHDA on the inhibition of mitophagy-related proteins (PINK1, parkin, VDAC1, and LC3-II) and anti-apoptotic Bcl-2 protein, as well as the induction of apoptotic-related proteins, and nuclear condensation. In conclusion, CA appears to counteract the neurotoxicity of 6-OHDA by activating PINK1/parkin-mediated mitophagy.

1. Introduction

Parkinson's disease (PD), a neurodegenerative movement disorder, progressively destroys the dopamine-containing neurons of the substantia nigra pars compacta. Mutation of PD-linked genes, such as *DJ-1*, *parkin*, or *Phosphatase and tensin homologue (PTEN)-induced kinase 1* (PINK1), is linked to the loss of dopaminergic neurons (Belin and Westerlund, 2008; Schapira, 2008). The brains of patients with PD show injured mitochondria and Lewy bodies, which contain aggregated proteins (Goedert, 2001; Shults, 2006). Autophagy is responsible for the degradation of aggregated and long-lived proteins, as well as impaired organelles (Levine and Klionsky, 2004). In particular, the removal of injured mitochondria through the process of autophagy is called "mitophagy" (Lemasters, 2005). During the mitophagy process, injured mitochondria are enveloped in the autophagosomal membrane to develop into the autophagosome and then fused with the lysosomes, leading to their degradation by hydroxylases (Zhang et al., 2010). The down-regulation of mitophagy mechanism causes injured mitochondria-triggered cellular toxicity. However, the up-regulation of mitophagy mechanism has been known to regulate by PINK1 and parkin (Okatsu et al., 2015). Therefore, gaining insight into the route of PINK1/parkin/mitophagy may assist us in understanding the pathogenic signaling pathways of PD.

The PINK1 gene is composed of a serine/threonine kinase domain and a N-terminal mitochondrial localization sequence (Valente et al., 2004). The physiologic roles of PINK1 are to regulate mitochondrial quality, morphology, and function (Valente et al., 2004). PINK1-deficient mice show inhibited mitochondrial respiration, abnormal mitochondrial morphology, and oxidative stress (Gispert et al., 2009). In addition to the effect of PINK1 in mitochondrial functions, it is also responsible for controlling cell survival. Overexpression of PINK1 in SH-SY5Y cells alleviates staurosporine-induced apoptosis (Petit et al., 2005). However, knockdown of PINK1 increases 1-methyl-4-phenylpyridinium (MPP^+) or rotenone-caused cytotoxicity and reduces the survival of SH-SY5Y cells (Deng et al., 2005). Moreover, in PINK1-deficient mice, the dopaminergic neurons of the substantia nigra pars compacta are more sensitive to MPTP-induced neuronal death (Haque et al., 2012).

The importance of PINK1 in neuroprotection is further supported because of its critical role in mitophagy (Geisler et al., 2010). In

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Abbreviations

AVO	Acidic vesicular organelles
CA	Carnosic acid
CCCP	Carbonyl cyanide-m-chlorophenylhydrazone
DiCO6	3, 3-Dihexyloxacarbocyanine iodide
DMSO	Dimethyl sulfoxide
MMP	Mitochondrial membrane potential
MPP+	1-methyl-4 phenylpyridinium
MPTP	1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine
6-OHDA	6-Hydroxydopamine
PARP	Poly ADP-ribose polymerase
PD	Parkinson's disease
PINK1	Phosphatase and tensin homologue (PTEN)-induced kinase 1
PMI	1-(3-iodo-phenyl)-4-(3-nitrophenyl)-1, 2, 3-triazole
siRNA	Small interfering RNA
VDAC1	Voltage-dependent anion channel 1

response to depolarization of the mitochondrial membrane potential (MMP) after carbonyl cyanide-*m*-chlorophenylhydrazone (CCCP) treatment, PINK1 accumulates on the outer membrane of depolarized mitochondria, and recruits parkin, an E3 ubiquitin ligase, to the surface of depolarized mitochondria (Kim et al., 2008). The voltage-dependent anion channel 1 (VDAC1), a substrate of parkin, is ubiquitinated by parkin. Then, the ubiquitination of VDAC1 recruits p62/SQSTM1/sequestosome-1 to the clustered mitochondria, resulting in damaged mitochondria for degradation by lysosomes (Geisler et al., 2010). By contrast, a lack of PINK1 or parkin leads to impairment of autophagy and mitochondrial dysregulation (Geisler et al., 2010). A study also indicated that knockout of parkin in *Drosophila* reduced mitochondrial proteins turnover is associated with the impairment of mitophagy by parkin.

(Vincow et al., 2013). Moreover, PINK1 and parkin deficiency in the midbrain of mice decreases mitochondrial respiration, mass, and dopaminergic neuronal survival (Lee et al., 2017; Stevens et al., 2015). The up-regulation of mitophagy by PINK1 and parkin is necessary for mitochondrial quality control.

Carnosic acid (CA) is a diterpene compound in rosemary. It is known to improve neurotoxin-induced neurodegeneration in *in vitro* and *in vivo* models of PD (Lin et al., 2017; Wu et al., 2015). An animal study indicated that administration of CA to mice can ameliorate the cyanide-induced loss of neurons in the hippocampus, striatum, and cortex (Zhang et al., 2015). In our previous studies, administration of 6-hydroxydopamine (6-OHDA), a neural toxin, to rats increased lipid peroxidation and decreased the expression of tyrosine hydroxylase, but pretreatment with CA reversed these effects (Wu et al., 2015). We also showed that CA can protect SH-SY5Y cells against a 6-OHDA-induced increase in reactive oxygen species and apoptotic signaling cascade by up-regulating glutathione synthesis and glutathione *S*-transferase (Chen et al., 2012; Lin et al., 2014, 2017). Our recent study also suggested that the protective role of CA against the cytotoxicity of 6-OHDA is mediated via activation of the ubiquitin-proteasome system and the autophagy pathway. Our findings also show that the mechanisms of this activation are associated with parkin induction (Lin and Tsai, 2016, 2017). However, how the action of parkin and PINK1 in mitophagy is associated with the protective role of CA is not clear. This study thus explored whether the role of PINK1/parkin-mediated mitophagy is related to the neuroprotection of CA in response to 6-OHDA.

2. Materials and methods

2.1. Materials

DMEM was ordered from Gibco Laboratory (Gaithersburg, MD). Dimethyl sulfoxide (DMSO), Triton-X 100, and bisBenzimide H 33258 were ordered from Sigma Chemical Company (St. Louis, MO). Fetal bovine serum was ordered from Hyclone (Logan, UT). 3,3-Dihexyloxacarbocyanine iodide ($DiCO_6$) was ordered from Calbiochem (San Diego, CA). Parkin, caspase 3, poly ADP-ribose polymerase (PARP), cleaved caspase 3, cleaved PARP, and LC3 primary antibodies were ordered from Cell Signaling Technology (Beverly, MA). PINK1, VDAC1, and β-tubulin primary antibodies were ordered from Santa Cruz Biotechnology (Santa Cruz, CA). Ubiquitin primary antibody was ordered from Millipore (Temecula, CA). Goat anti-mouse IgG (H + L) secondary antibody-Alexa Fluor® 546 conjugate was purchased from Thermo Fisher Scientific (Rockford, IL).

2.2. Cell culture and treatment

The cell culture protocol was performed according to our earlier research (Chen et al., 2012). Briefly, SH-SY5Y cells were grown in DMEM medium with 10% fetal bovine serum and were then maintained using an incubator with a humidified atmosphere of 5% CO_2 at 37 °C. CA (A. G. Scientific, Inc., San Diego, CA) and 6-OHDA (Sigma Chemical Company, St. Louis, MO) were dissolved with DMSO. Cells were grown up to 80% confluence and were then cultured with CA (1 μM) for 18 h before exposure to 6-OHDA (100 μM) for the indicated time. DMSO (0.3%) was added to the control cells.

2.3. Measurement of mitochondrial membrane potential

The $DiCO_6$ dye was used to measure the MMP (Nam et al., 2015). After the cells were rinsed with phosphate-buffered saline, they were exposed to $DiCO_6$ dye (1 μM) in the incubator for 30 min. The fluorescence microscope was used to detect changes in MMP. The images were quantified for fluorescence intensity using Image-Pro Plus 6.0 (Media Cybernetics, Inc., Bethesda, MD).

2.4. Hoechst 33258 nuclear staining

The method of Hoechst 33258 nuclear staining was performed according to our earlier research (Chen et al., 2012). The fluorescence microscope was used to measure nuclear morphology. Fluorescence intensity was analyzed quantitatively by using Image-Pro Plus 6.0 (Media Cybernetics, Inc., Bethesda, MD).

2.5. Acidic vesicular organelle staining

The method of acidic vesicular organelle (AVO) staining was performed according to our earlier research (Lin and Tsai, 2017). After the medium was removed, cells were rinsed in phosphate-buffered saline and incubated with orange hydrochloride solution at 37 °C in the dark. After incubation, the formation of AVO was detected under the fluorescence microscope.

2.6. Immunofluorescence assay

Cells were fixed in phosphate-buffered saline with 4% paraformaldehyde and 2% sucrose for 15 min in the dark at 25 °C. After exposure to the permeabilization buffer (1% bovine serum albumin and 0.3% Triton-X 100) for 5 min on ice, cells were blocked with 3% bovine serum albumin for 30 min at 37 °C. Moreover, they were co-immunostained with the primary parkin antibody and MitoTracker green FM probe (purchased from Molecular Probes), a mitochondrial marker, overnight at 37 °C. The next day, goat anti-mouse IgG (H + L) secondary antibody-Alexa Fluor 546 conjugate (red fluorescence for

parkin) and Hoechst 33258 dye (blue fluorescence for nucleus) were added to the cells for 2 h at 37 °C. Finally, fluorescence intensity was detected under the fluorescence microscope.

2.7. Western blotting

The method was determined according to our earlier research (Lin et al., 2014). In brief, 10 μg protein of each lysate was subjected to 10% and 12.5% separating gels, respectively, and transferred to polyvinylidene fluoride membranes (Millipore, Bedford, MA). After blocking with 5% nonfat milk, all primary antibodies were used to incubate with blots overnight at 4 °C. Horseradish peroxidase-conjugated goat anti-rabbit IgG and rabbit anti-goat IgG (ordered from R&D Systems Inc., Minneapolis, MN), as well as anti-mouse IgG (all ordered from Santa Cruz Biotechnology, Santa Cruz, CA), served as secondary antibodies. The signaling on the blots was detected with an enhanced chemiluminescence kit (ordered from PerkinElmer Life Science, Boston, MA) using a luminescent image analyzer (LAS-4000, FUJIFILM, Japan).

2.8. Transient transfection of small interfering RNA

The method was performed as in Lin et al.'s study (Lin et al., 2014). The sequence of human PINK1 small interfering RNA (siRNA) was used: 5′-GAAAUCCGACAACAUCCUUUU-3'; human parkin siRNA was used: 5′-UUCGCAGGUGACUUUCCUCUGGUCA-3'.

Each siRNA sequence at the final concentration of 50 nM was individually transfected into the cells with Dharma FECT siRNA transfection reagent by the manufacturer's protocol (Thermo Fisher Scientific). After transfection for 24 h, cells were changed to fresh medium containing 1 μM CA for 18 h before being exposed to 100 μM 6-OHDA for 12 h (Western blotting) and 18 h (AVO and Hoechst 33258 staining).

2.9. Immunoprecipitation assay

The protocol was as described in our early research (Lin and Tsai, 2017). In brief, the primary VDAC1 antibody reacted with 60 μg of total protein overnight at 4°C followed by mixture with Protein A-Sepharose beads (0.1 g/L) on ice for 4 h. Each lysate with VDAC1 primary antibody was centrifuged at 16,000 × *g* for 10 min at 4 °C, and each pellet was rinsed twice in immunoprecipitation buffer. Finally, each immunoprecipitation complex was boiled and subjected to Western blotting.

2.10. Statistical analysis

Results were analyzed for statistical significance by use of commercially available software (SAS Institute Inc., Cary, NC) with one-way ANOVA with Tukey's test. The results of Figs. 1 and 3 were analyzed by using Duncan's test. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Pretreating cells with CA suppresses 6-OHDA-elicited mitochondrial impairment

To confirm whether CA influenced 6-OHDA-induced mitochondrial impairment, we used $DiCO_6$ dye to detect MMP. In contrast with control cells, cells incubated with 6-OHDA had a reduced intensity of green fluorescence; however, this fluorescence intensity was improved in CA-pretreated cells (Fig. 1A). In mitochondria damaged because of a low MMP, *cytochrome c* is released from the mitochondria to the cytosol, and cytosolic *cytochrome c* activates the downstream apoptotic cascade (Wang and Youle, 2009). As shown in Fig. 1B, the protein expression of cytosolic *cytochrome c* was increased as a result of 6-OHDA treatment, but this protein induction was reduced after CA pretreatment.

3.2. Pretreating cells with CA recruits parkin to damaged mitochondria

Parkin is recruited by PINK1 to damaged mitochondria, where it ubiquitinates the VDAC1 protein. The damaged mitochondria are degraded via the activation of mitophagy (Geisler et al., 2010). We used an immunofluorescence assay to determine whether parkin was recruited into the mitochondria. As shown in Fig. 2, 6-OHDA treatment decreased red (parkin) and MitoTracker green FM (mitochondria) fluorescences, indicating that 6-OHDA reduced parkin protein and impaired the mitochondria. However, in cells pretreated with CA, the two fluorescent signals were significantly co-localized, indicating that CA pretreatment increased parkin protein and enhanced parkin to recruit damaged mitochondria.

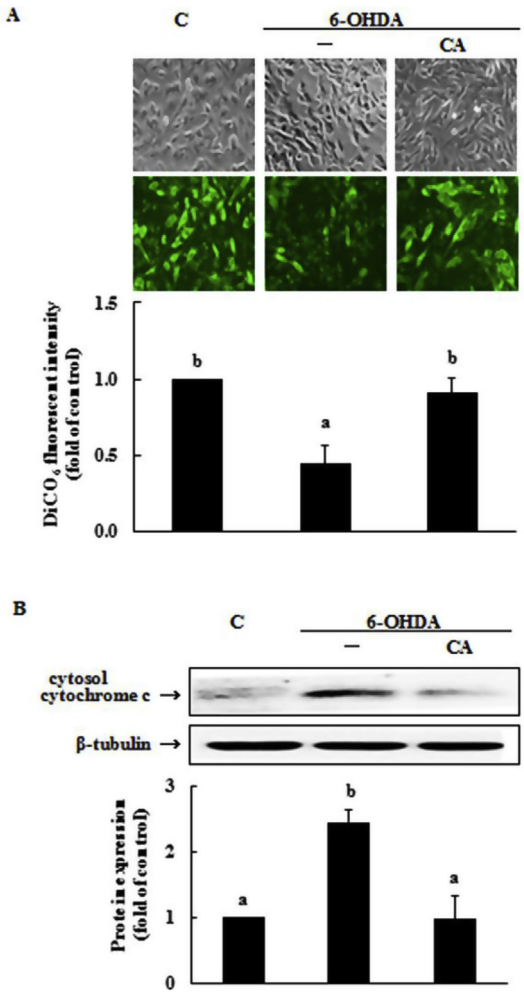

Fig. 1. Effect of CA on 6-OHDA-induced MMP loss and cytosolic *cytochrome c* level. After CA (1 μM) pretreatment, 6-OHDA (100 μM) was added to cells at 3 h (MMP) or 12 h (cytosolic *cytochrome c* level). Cells treated with DMSO (0.3%) were considered the control group (C). (A) $DiCO_6$ dye was used to determine the MMP and then visualized by fluorescence microscope. Phase contrast views are presented as the upper images. Fluorescent views are presented as the lower images. (B) Cytosolic *cytochrome c* protein and β–tubulin, a loading control, are measured for Western blotting. The fold of the control group was defined as 1.0. A representative image was chosen from three individual replicates. Values are expressed as means ± SD of three individual replicates. Different letters indicate statistically significant differences ($p < 0.05$).

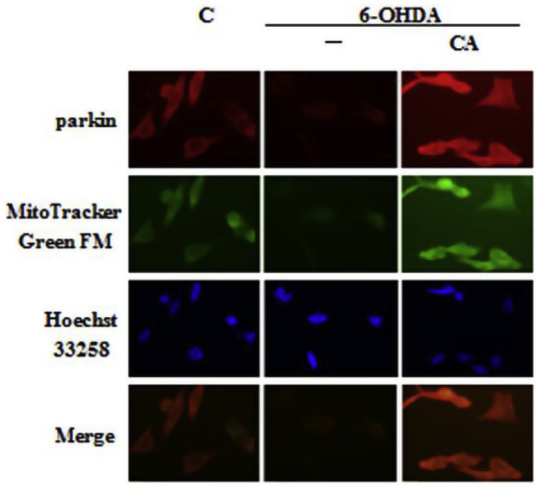

Fig. 2. Effect of treatment with CA and 6-OHDA on the translocation of parkin to damaged mitochondria. Before exposure to 6-OHDA (100 μM) for 12 h, cells were cultured with fresh medium containing CA (1 μM) for 18 h. Cells treated with DMSO (0.3%) were considered the control group (C). Cells were co-immunostained using a primary antibody to parkin (red), MitoTracker Green FM as a mitochondrial marker (green), and Hoechst 33258 as a nuclear morphology marker (blue). The fluorescence microscope was used to visualize fluorescences. Fluorescence views of both parkin and MitoTracker Green FM were merged. A representative image was chosen from three individual replicates. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.3. Pretreating cells with CA increases the ubiquitination of VDAC1

As shown by immunoblotting, cells treated with 6-OHDA had markedly decreased expression of VDAC1 protein, but this reduction in protein expression was reversed by CA pretreatment (Fig. 3A). Moreover, we used the immunoprecipitation assay to examine the ubiquitination of VDAC1 after exposure to CA and 6-OHDA. After immunoprecipitation of cell lysates with a VDAC1 primary antibody, the immunoprecipitation complexes were subjected to immunoblotting. As shown in Fig. 3B, exposure to 6-OHDA reduced the interaction of ubiquitinated protein with VDAC1. Compared with 6-OHDA treatment, CA pretreatment caused an increase in the interaction of ubiquitinated proteins with VDAC1.

3.4. PINK1 and parkin are required for activation of mitophagy by CA pretreatment

In our previous study, we showed that pretreating cells with CA improved the inhibition of PINK1 protein by 6-OHDA (Lin and Tsai, 2016). PINK1 is upstream of parkin and functions to remove damaged mitochondria by mitophagy (Geisler et al., 2010). Cells were transfected with PINK1 siRNA to confirm whether PINK1 is involved in the mitophagy by CA. After transfection with nontargeting control siRNA, 6-OHDA decreased PINK1, parkin, and VDAC1 protein expression. The inhibition of these proteins by 6-OHDA was significantly reversed by CA pretreatment. Treatment with 6-OHDA for 12 h did not affect the ratio of LC3-II to LC3-I; however, CA pretreatment increased this ratio by nearly 80%. In contrast to cells transfected with PINK1 siRNA, in cells pretreated with CA, the suppression of these proteins upon 6-OHDA treatment was no longer markedly reversed (Fig. 4).

To determine whether the formation of autophagic vacuoles was regulated by PINK1 and parkin, PINK1 and parkin siRNAs were individually transfected into cells. As shown by AVO staining, 6-OHDA treatment decreased the fluorescence intensity of AVO. Pretreatment with CA improved 6-OHDA-induced suppression of fluorescence intensity of AVO. However, the effect of CA on AVO fluorescence

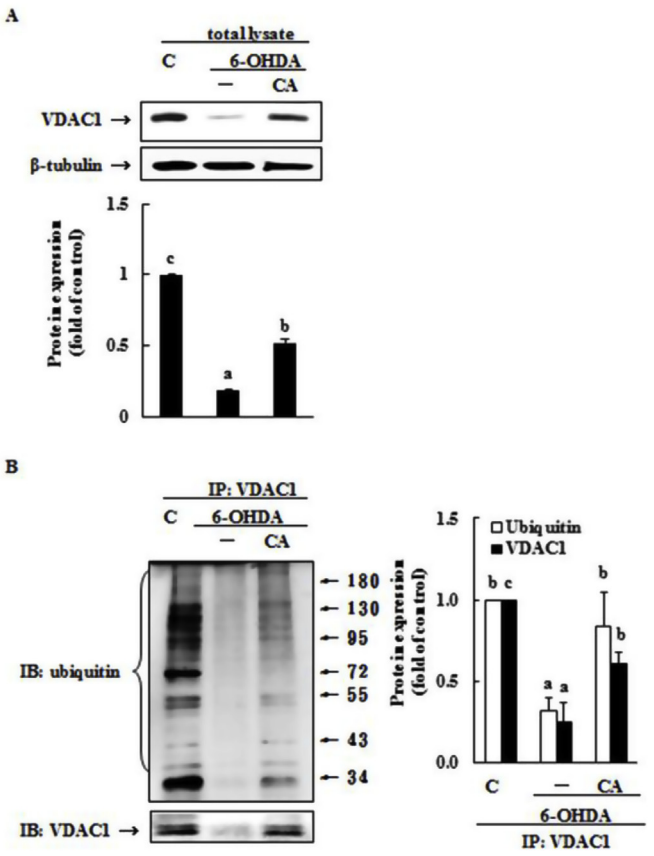

Fig. 3. Effect of CA on the ubiquitination of VDAC1 by 6-OHDA. Before cells were exposed to 6-OHDA (100 μM) for 12 h, they were cultured with fresh medium, including CA (1 μM) for 18 h. Cells treated with DMSO (0.3%) were considered the control group (C). (A) Protein expression of VDAC1 and β-tubulin, a loading control, was measured for Western blotting. (B) Total protein of cell lysates were subjected to immunoprecipitation with a primary VDAC1 antibody, and immunoprecipitated complexes were measured for Western blotting. The fold of the control group was defined as 1.0. A representative image was chosen from three individual replicates. Values are expressed as means ± SD of three individual replicates. Different letters indicate statistically significant differences ($p < 0.05$).

intensity was decreased after PINK1 or parkin siRNA transfection (Fig. 5). These results showed that PINK1 and parkin siRNA inhibited the effect of CA to alleviate 6-OHDA-reduced generation of autophagic vacuoles.

3.5. PINK1 siRNA inhibits the anti-apoptotic ability of CA

To investigate whether PINK1 is linked to the effect of CA to attenuate 6-OHDA-induced apoptosis, we measured the apoptotic-related proteins and nuclear condensation in response to PINK siRNA. After transfection of nontargeting control siRNA, exposure to 6-OHDA reduced the expression of anti-apoptotic Bcl-2 protein and elevated the cleavage of caspase 3 and PARP. Pretreating cells with CA counteracted these effects. Treatment with PINK1 siRNA decreased the ability of CA to inhibit the effect of 6-OHDA on the induction of apoptosis-related proteins (Fig. 6A). The results of Hoechst 33258 staining were consistent with the immunoblotting results, as shown in Fig. 6B. Exposure to 6-OHDA in the nontargeting control siRNA group induced the intensity of Hoechst 33258 fluorescence, suggesting that 6-OHDA treatment increased nuclear condensation, leading to the induction of apoptosis. However, CA pretreatment attenuated the effect of 6-OHDA on nuclear condensation. Moreover, after PINK1 siRNA transfection, CA could no longer significantly inhibit the increase in nuclear

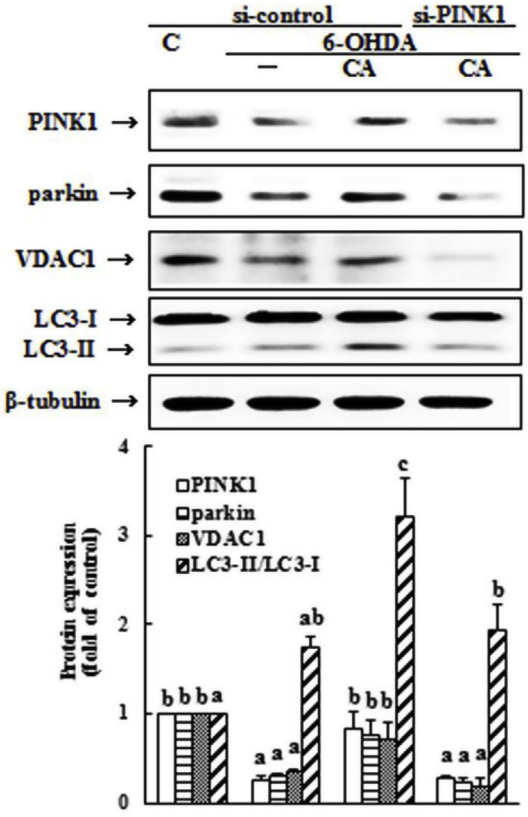

Fig. 4. The role of PINK1 siRNA by CA on PINK1, parkin, and VDAC1 proteins, and LC3-II/LC3-I ratio expression in the 6-OHDA group. Nontargeting control siRNA and PINK siRNA were transfected into cells for 24 h and were then cultured with CA (1 μM) for 18 h before exposure to 6-OHDA (100 μM) for 12 h. DMSO (0.3%) was added to the cells with nontargeting control siRNA (si-control, C). Western blotting was used to determine protein expression, including PINK1, parkin, VDAC1, LC3-I, LC3-II, and β–tubulin. The fold of control cells with nontargeting control siRNA was defined as 1.0. A representative image was chosen from three individual replicates. Values are expressed as means ± SD of three individual replicates. Different letters indicate statistically significant differences ($p < 0.05$).

condensation by 6-OHDA. It is thus likely that PINK1 is necessary for CA to reduce 6-OHDA-triggered cell death.

4. Discussion

Mitochondria are known as the powerhouse of the cell. They are responsible for supplying cellular energy, cell differentiation, cell growth, and cell death (McBride et al., 2006). Mitochondrial dysfunction causes neuronal loss, resulting in neurodegenerative diseases, including PD (Hu and Wang, 2016). Therefore, ways to reduce defective mitochondria are regarded as an important means of maintaining neuronal homeostasis. A report by de Oliveira et al. suggested that culturing SH-SY5Y cells with paraquat, a herbicide, decreases the enzyme activities of mitochondrial respiratory chain complexes I and V, as well as the production of ATP. However, pretreatment of cells with CA could protect against paraquat-induced mitochondrial impairment (de Oliveira et al., 2016). Another report indicated that 6-OHDA exposure induces neuronal damage by elevating MMP collapse, increasing the cytosolic *cytochrome c* level, and activating the mitochondria-mediated apoptotic cascade. By contrast, a phytoestrogen β-ecdysterone alleviates 6-OHDA-triggered mitochondria-mediated apoptosis (Pan et al., 2016). In our previous research, we showed that neuroprotection by CA is linked to reduced levels of apoptosis-related proteins in the TGF-β signaling pathway through ubiquitin-proteasome system-mediated degradation by parkin (Fu et al., 2018). In the present study, we showed that PINK1 activates parkin to ubiquitinate VDAC1 and enhance mitophagy.

In this study, exposure of cells to 6-OHDA disrupted the MMP and induced the expression of cytosolic *cytochrome c*; however, these effects of 6-OHDA were alleviated by pretreatment with CA (Fig. 1). We assumed that the prevention of 6-OHDA-caused mitochondrial impairment by CA was linked to mitochondrial clearance. Mitophagy is the specific elimination of impaired mitochondria by lysosomes (Lemasters, 2005). It is known that the activation of mitophagy is mediated via PINK1 and parkin. Moreover, research by Geisler et al. showed that VDAC1 is required for PINK1/parkin-mediated mitophagy (Geisler et al., 2010). VDAC1 is localized on the outer mitochondrial membrane and is involved in cellular metabolism, the calcium ion transport channel, cell survival, and apoptosis (Shoshan-Barmatz et al., 2018). In addition to these functions, VDAC1 serves as a candidate target for mitophagic processes (Geisler et al., 2010). Because VDAC1 is a mitochondrial substrate of parkin, parkin increases the ubiquitination of VDAC1 after mitochondrial depolarization. Moreover, the autophagic adaptor protein p62 is recruited to the mitochondrial clusters by VDAC1, leading to mitochondrial degradation through lysosomes (Geisler et al., 2010). Watanabe's study also indicated that HL-1 mouse

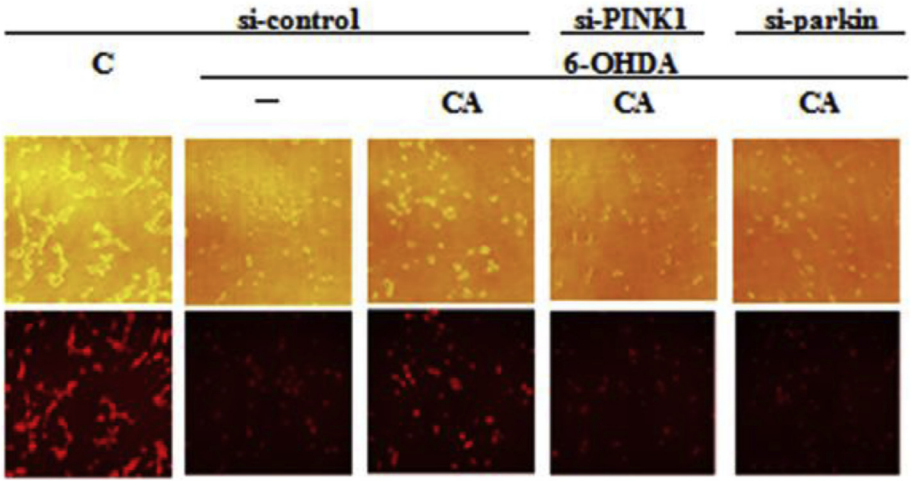

Fig. 5. Effect of treatment with parkin and PINK1 siRNAs on the ability of CA to influence autophagic vacuole generation after 6-OHDA treatment. Cells were transfected with parkin, PINK1, and nontargeting control siRNAs for 24 h. They were cultured with CA (1 μM) for 18 h before exposure to 6-OHDA (100 μM) for 18 h. DMSO (0.3%) was added to the cells with nontargeting control siRNA (si-control, C). The fluorescence microscope was used to visualize the dye fluorescence. Phase contrast views are presented as the upper images. Fluorescent views are presented as the lower images. A representative image was chosen from three individual replicates. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

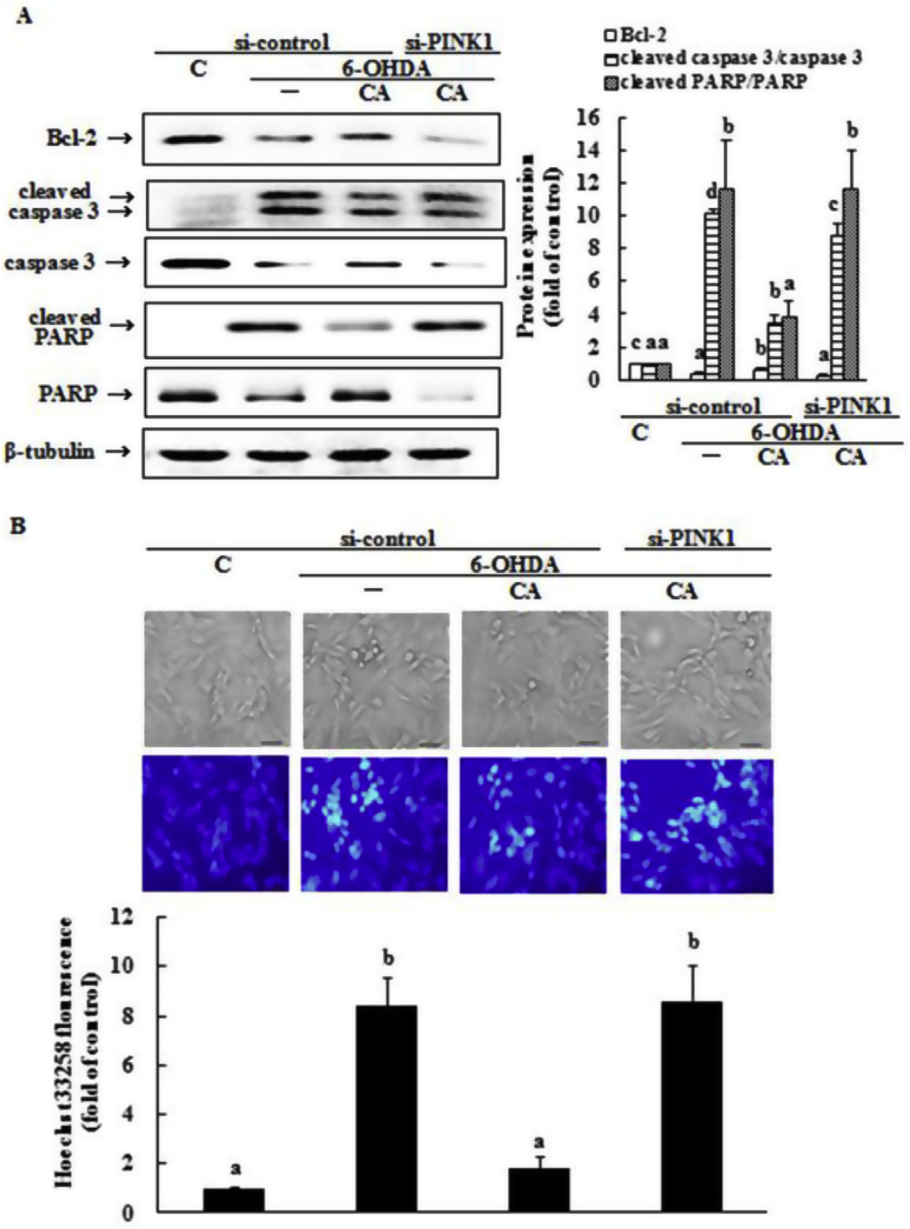

Fig. 6. Effect of treatment with PINK1 siRNA on the ability of CA to influence the levels of apoptotic-related proteins and nuclear condensation after 6-OHDA treatment. Cells were transfected with parkin, PINK1, and nontargeting control siRNAs for 24 h. They were cultured with CA (1 μM) for 18 h before exposure to 6-OHDA (100 μM) for 12 h (Western blotting) and 18 h (Hoechst 33258 nuclear staining). DMSO (0.3%) was added to the cells with nontargeting control siRNA (si-control, C). (A) Apoptotic-related proteins and β–tubulin, a loading control, were detected for Western blotting. (B) The fluorescence of Hoechst 33258 dye was visualized using a fluorescence microscope. Phase contrast views are presented as the upper images. Fluorescent views are presented as the lower images. The fluorescence intensity of Hoechst 33258 dye was quantified using Image-Pro Plus 6.0. The fold of control cells with nontargeting control siRNA was defined as 1.0. A representative image was chosen from three individual replicates. Values are expressed as means ± SD of three individual replicates. Different letters indicate statistically significant differences ($p < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

atrial cardiomyocytes treated with arsenic trioxide induce the translocation of parkin into the outer membrane of depolarized mitochondria to promote the ubiquitination of VDAC1 and then remove the injured mitochondria (Watanabe et al., 2014). In contrast, mitochondria fail to be removed after transfection with pathogenic parkin mutants or VDAC1 siRNA (Geisler et al., 2010). Our immunofluorescence assay showed that CA pretreatment counteracted the effect of 6-OHDA on the red (parkin) and MitoTracker green FM (mitochondria) fluorescence, resulting in significant co-localization of the two fluorescences. This result implied the recruitment of parkin to injured mitochondria by CA (Fig. 2). After immunoprecipitation with the VDAC1 antibody, pretreatment with CA increased the immunoprecipitation of ubiquitin compared to 6-OHDA treatment (Fig. 3). It is likely that CA promotes the conjugation of ubiquitinated proteins with VDAC1 and prevents 6-OHDA from inhibiting the mitophagic processes by enhancing the ubiquitination of VDAC1. Additionally, by AVO staining, we showed that CA could reverse the reduction in formation of autophagic vacuoles by 6-OHDA treatment. However, knockdown of parkin inhibited the effect of CA (Fig. 5). These data are also supported by our early research, indicating that parkin siRNA attenuates the ability of CA to improve 6-OHDA-reduced autophagic processes (Lin and Tsai, 2017). Therefore, we showed that CA increases the clearance of impaired mitochondria by activating parkin-mediated mitophagy.

PINK1 plays a vital role in maintaining mitochondrial homeostasis and neuronal survival (Valente et al., 2004). Knockdown of PINK1 in SH-SY5Y cells reduces MMP, mitochondrial respiration, ATP synthesis, and cell survival (van der Merwe et al., 2017). Overexpression of PINK1 protects against the proteasome inhibitor MG-132-induced MMP loss and neuronal death (Valente et al., 2004). This is supported by our results in the present study showing that CA increased the protein expression of PINK1 to decrease 6-OHDA-induced apoptosis-related proteins and nuclear condensation. However, transfection of cells with PINK1 siRNA inhibited the ability of CA to reverse these findings by 6-OHDA (Fig. 6). In addition, PINK1 is upstream of parkin and it activates mitophagy by recruiting parkin (Okatsu et al., 2015). Silencing of PINK1 in SH-SY5Y cells transfected with PINK1 siRNA inhibited the recruitment of parkin to the depolarized mitochondria after CCCP treatment for 2 h and the removal of injured mitochondria within 24 h.

Moreover, in the absence of endogenous PINK1, retransfection of cells with V5-tagged wild-type PINK1 could attenuate CCCP-induced parkin recruitment and injured mitochondrial degradation (Geisler et al., 2010). To explore whether PINK1 is associated with the induction of mitophagy and the inhibition of apoptosis by CA, we used PINK1 siRNA. As shown by immunoblotting and AVO staining, suppression of PINK1 abolished the reversal of CA to inhibit 6-OHDA-reduced expression of parkin and VDAC1, as well as the generation of autophagic vacuoles (Figs. 4 and 5). The induction of the ratio of LC3-II to LC3-I caused by CA was attenuated in cells treated with PINK1 siRNA (Fig. 4). It is possible that the enhancement of PINK1 by CA is required for the induction of mitophagy by parkin to reduce 6-OHDA-induced apoptosis. Improving mitochondrial function may serve as an additional therapeutic approach in the treatment of PD. A report by East et al. indicated that the natural compound 1-(3-iodo-phenyl)-4-(3-nitrophenyl)-1,2,3-triazole (PMI) can activate mitophagy without the toxicity connected with MMP collapse (East et al., 2014). Like PMI, CA showed no toxicity in the present study and removed impaired mitochondria by activating mitophagy. Because CA can cross the blood-brain barrier, it may be suitable for use as a neuroprotection compound to clear damaged mitochondrial and for PD prevention.

5. Conclusion

In this study, exposure of SH-SY5Y cells to 6-OHDA induces the apoptotic pathway, whereas pretreatment with CA inhibits this signaling. In the presence of CA, the degradation of impaired mitochondria by mitophagy is connected to the recruitment of parkin into impaired mitochondria and the ubiquitination of VDAC1. Moreover, the neuroprotective role of PINK1 is necessary for the parkin-mediated mitophagy and cell survival after CA pretreatment. Taken together, our results suggest that CA protects SH-SY5Y cells against mitochondrial impairment induced by 6-OHDA by activating the PINK1/parkin/mitophagy pathway. The mechanism of CA in mitophagy suggests a therapeutic application for CA in the treatment of PD.

Conflicts of interest

The authors declare that there are no conflicts of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fct.2019.01.027.

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