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L-DOPA-induced neurotoxicity is reduced by the activation of the PI3K signaling pathway

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ABSTRACT

L-3,4-Dihydroxyphenylalanine (L-DOPA) is one of the most important drugs for the treatment of Parkinson's disease (PD). Although neurotoxicity of L-DOPA remains controversial, there are many reports suggesting that L-DOPA causes neuronal death. We investigated whether the neurotoxic effect of L-DOPA could be inhibited by the activation of the phosphatidylinositol 3-kinase (PI3K) pathway. Cell counting kit-8, trypan blue staining, and DAPI staining all showed that L-DOPA decreased nPC12 cell viability at high concentrations. However, combined treatment with the PI3K activator and L-DOPA significantly increased the viability of nPC12 cells when compared with treatment with L-DOPA only. Phosphorylated Akt (Ser473), phosphorylated glycogen synthase kinase-3β (GSK-3β) (Ser9), and heat shock transcription factor-1, which are survival-related signaling proteins, were decreased in nPC12 cells treated with 200 μM L-DOPA, but were significantly increased with combined treatment with the PI3K activator in a concentration-dependent manner. However, treatment of L-DOPA significantly increased expressions of cytosolic cytochrome *c* and cleaved caspase-3, which are death-related signaling proteins, in nPC12 cells, but combined treatment with the PI3K activator reduced those expressions. To confirm whether the effect of the PI3K activator is associated with direct activation of PI3K, LY294002, a PI3K inhibitor, was used to pretreat the nPC12 cells prior to combined treatment with the PI3K activator and L-DOPA. The protective effect of the PI3K activator was almost completely blocked. Together, these results suggest that L-DOPA neurotoxicity can be prevented by PI3K activation.

1. Introduction

L-3,4-Dihydroxyphenylalanine (L-DOPA) is the gold standard for the treatment of Parkinson's disease (PD), which is characterized by resting tremors, rigidity, bradykinesia and postural instability due to a dopamine deficiency. Since L-DOPA was first used in clinical trials in the 1960s, there have been many reports documenting its therapeutic success in the treatment of symptomatic PD (Cotzias et al., 1967; Fehling, 1966). However, despite the obvious benefits of L-DOPA treatment, a potential role of L-DOPA in neurotoxicity has been suggested. Although there is significant controversy regarding the neurotoxicity of L-DOPA, a number of papers have suggested that L-DOPA promotes neurodegeneration and accelerates progression of PD (Chen et al., 2003; Koh et al., in press; Lin and Kang, 2005; Maharaj et al., 2005; Muller et al., 2004; Soliman et al., 2002; Du et al., 2009). The best known potential mechanisms of L-DOPA neurotoxicity involve oxidative stress, including nitrosative stress and increased generation of neurotoxins; oxidation of L-DOPA to quinone and semiquinone: mitochondrial dysfunction; and genomic DNA damage (Chen et al., 2003; Maharaj et al., 2005; Muller et al., 2004; Soliman et al., 2002). However, there is no report about the effects of L-DOPA on intracellular survival signaling proteins including phosphatidylinositol 3-kinase (PI3K).

Several kinds of neuroprotectants have been reported to prevent neuronal death by activating the PI3K signaling pathway (Crowder and Freeman, 1998; Li et al., 2003; Philpott et al., 1997). For example, nerve growth factor, vascular endothelial growth factor, and insulin-like growth factor-1 (IGF-1) can activate PI3K (Crowder and Freeman, 1998; Li et al., 2003; Philpott et al., 1997). The activated PI3K then phosphorylates the serine/threonine kinase Akt (protein kinase B), the main PI3K downstream effecter (Klippel et al., 1998; Koh et al., 2005a,b). Considering that the PI3K signaling pathway

Abbreviations: CCK-8, cell counting kit-8; DAPI, DNA-specific fluorochrome 4′,6-diamidino-2-phenylindole dihydrochloride; GSK-3β, glycogen synthase kinase-3β; HSTF-1, heat shock transcription factor-1; IGF-1, insulin-like growth factor-1; IR, immunoreactivity; L-DOPA, L-3,4-dihydroxyphenylalanine; nPC12, neuronally differentiated PC12; pAkt, phosphorylated Akt; PD, Parkinson's disease; pGKS-3β, phosphorylated GSK-3β; PI3K, phosphatidylinositol 3-kinase; PMSF, phenylmethylsulfonyl fluoride; SCEB, sucrose-supplemented cell extract buffer; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TBST, Tris buffered saline containing 0.05% Tween-20.

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plays a central role in neuronal survival (Crowder and Freeman, 1998; Koh et al., 2003a,b; Philpott et al., 1997), we can hypothesize that activation of the PI3K signaling pathway might be helpful in preventing L-DOPA-induced neurotoxicity.

In this study, therefore, we investigated whether L-DOPA affects the PI3K signaling pathway and whether direct PI3K activation can reduce L-DOPA-induced neurotoxicity.

2. Materials and methods

2.1. Materials

L-DOPA was provided by Myung In Pharmacy, Seoul, Korea, and its purity was more than 97%. The PI3K activator was purchased from Santa Cruz Biotech (Delaware, CA, USA) and is a peptide with the sequence KKHTDDGYMPMSPGVA and a molecular weight of 1732.8 Da. The tyrosine phosphorylated version of this peptide binds to the PI3Kinase SH2 domain activating the enzyme (Garcia et al., 1993; Rordorf-Nikolic et al., 1995; Shoelson et al., 1992). Before use in the experiments, these drugs were dissolved in distilled water and further diluted with culture medium to yield the desired final concentrations. LY294002, a PI3K inhibitor, was purchased from Sigma (Saint Louis, MO, USA) to directly block PI3K.

2.2. Cell culture, induction of differentiation, and treatment

Undifferentiated PC12 cells (ATCC, Rockville, MD, USA) were maintained in logarithmic-phase growth on poly-L-lysine (Sigma, Saint Louis, MO, USA)-precoated 100 mm dishes (Corning, PA, USA) in RPMI 1640 medium (GIBCO, Grand Island, NY, USA) containing 10% heat-inactivated horse serum, 5% heat-inactivated fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cultures were incubated at 37 °C in a humidified atmosphere with 5% CO_2. The cultures were grown to 50% confluence and were harvested in Ca^{2+}/Mg^{2+}-free Hank's balanced salt solution containing 1 mM EDTA. Cells were plated at a density of 1×10^6 cells/100 mm dish and incubated for 24 h. For neuronal differentiation, PC12 cells were serum-starved for 12 h (RPMI 1640 medium containing 100 units/ml penicillin and 100 μg/ml streptomycin without horse serum or fetal bovine serum); thereafter, the cells were maintained in serum-free medium. After two days the media was replaced with fresh serum-free media. On day three, NGF (50 ng/ml, Sigma, Saint Louis, MO, USA) was added to the medium, and the cultures were maintained for an additional three days. The extent of cell differentiation was determined by examination under an Image Gauge photographing microscope (version 3.12, Fuji Photo Co., Tokyo, Japan). Randomly selected fields containing approximately 100 cells were photographed, and the numbers of undifferentiated and differentiated cells were counted. Cells having neurites greater than two cell body diameters in length were considered differentiated (Koh et al., 2003a,b).

Different concentrations of L-DOPA were tested to assess its effect on neuronal cell viability. After neuronally differentiated PC12 (nPC12) cells were incubated with L-DOPA [0 (control), 5, 10, 20, 50, 100, 200, and 500 μM] for 24 h, each plate was washed, and cell viability was measured using a cell counting kit-8 (CCK-8) and trypan blue stain.

To evaluate the role of PI3K activation in L-DOPA-induced neurotoxicity, nPC12 cells were incubated in the presence of 200 μM L-DOPA together with several concentrations of PI3K activator (0, 10^{-2}, 10^{-1}, 10^{0}, 10^{1}, and 10^{2} μg/ml) for 24 h.

To investigate whether the effects seen were due to direct PI3K activation, cells were treated with 100 μM LY294002 (PI3K inhibitor; Sigma, Saint Louis, MO, USA) along with the activator; cell viability was then evaluated.

To estimate the alteration of intracellular signals such as phosphorylated Akt (pAkt) (Ser473), phosphorylated GSK-3β (pGKS-3β) (Ser9), heat shock transcription factor-1 (HSTF-1), cytosolic cytochrome *c*, and the cleaved form of caspase-3 by L-DOPA and PI3K activator, nPC12 cells were harvested for 24 h after treatment with several conditions and immediately used for immunoblotting.

2.3. CCK-8 assay and trypan blue staining measure cell viability

The CCK-8 was used to count living cells by combining WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] and 1-methoxy PMS. Briefly, 10 μl of kit reagent was added to the cells treated as described above in 96-well plates and incubated for 3 h. Cell viability was assessed using the ELISA plate reader at 450 nm. All results were normalized to OD values measured from an identically conditioned well without cell culture (Noh et al., 2009). For trypan blue staining, 10 μl of trypan blue solution (BMS, Seoul, Korea) was incubated for 2 min with 10 μl of cells from each sample. Unstained live cells were counted on a hemacytometer (Noh et al., 2009).

2.4. DAPI staining to assess apoptosis

DAPI staining was performed to evaluate the status of apoptosis as follows. nPC12 cells were incubated for 24 h with one of the three treatments: (1) without L-DOPA or PI3K activator, (2) with only 200 μM L-DOPA, and (3) with both 200 μM L-DOPA and 1, 10, or 100 μg/ml PI3K activator. All samples were centrifuged at

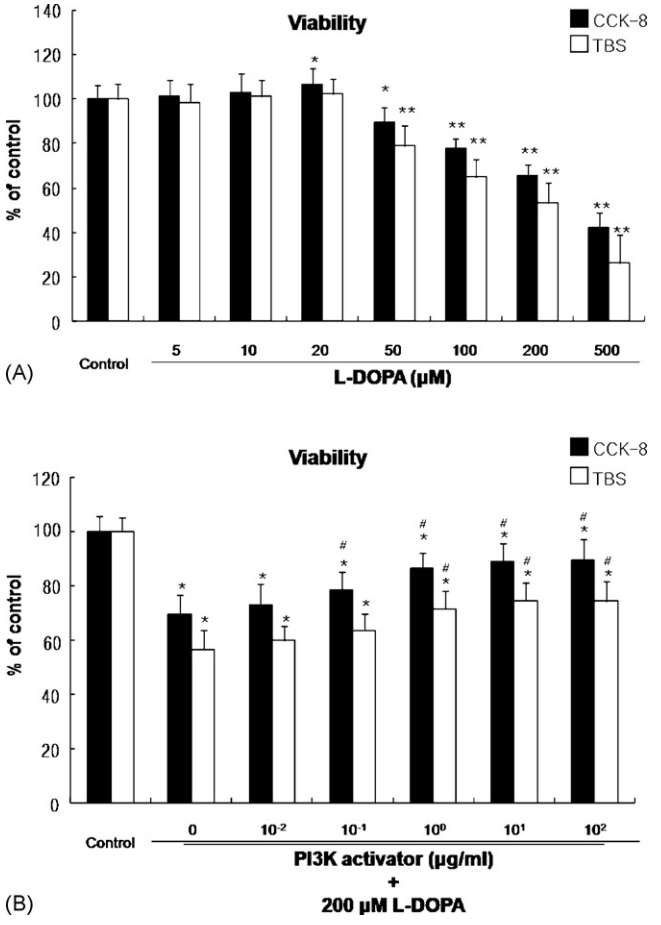

Fig. 1. Measurement of neuronally differentiated PC12 cell viability by CCK-8 assay and trypan blue staining. Data are presented as means (% of control) ± SEM from five or more independent experiments. Cell viability was significantly decreased at high concentrations (A). Decreased viability induced by 200 μM L-DOPA was effectively restored with the combined treatment of a PI3K activator (B) in a concentration-dependent manner. Each treatment group was compared with the other groups using Duncan's multiple range test after one-way ANOVA. $^{*}p < 0.05$ when compared with the control group. $^{\#}p < 0.05$ when compared with the group treated with 200 μM L-DOPA only (lane 0).

2000 × *g* for 2 min, the supernatants were discarded, and 4% neutral buffered formalin (100 μl) was added to each cell pellet. A 50 μl aliquot of the cell suspension was applied to a glass slide and dried at room temperature. The fixed cells were then washed with PBS, air dried, and stained with the DNA-specific fluorochrome 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI, Sigma, Saint Louis, MO, USA) for 20 min. The adherent cells were then rinsed with PBS, air dried, and mounted with 90% glycerol. The slides were observed under an Olympus fluorescence microscope (Jiang et al., 2000). A total of 300 cells were counted to determine the percentage of cells exhibiting the morphological hallmarks of apoptosis such as DNA fragmentation, nuclear condensation, or segmentation.

2.5. Western blot analysis

pAkt (Ser473), pGSK-3β (Ser9), HSTF-1, cytosolic cytochrome *c*, and the cleaved form of caspase-3 were analyzed by Western blot. Briefly, 5×10^6 cells were collected at 24 h post-differentiation, washed twice in cold PBS and incubated for 10 min on ice in lysis buffer [50 mM Tris (pH 8.0), 150 mM NaCl, 0.02% sodium azide, 0.2% SDS, 100 μg/ml phenylmethylsulfonyl fluoride (PMSF), 50 μl/ml aprotinin, 1% igepal 630, 100 mM NaF, 0.5% sodium deoxycholate, 0.5 mM EDTA, 0.1 mM EGTA]. Cell lysates were centrifuged at 10,000 × *g* for 20 min at 4 °C, and the supernatants were used to evaluate pAkt (Ser473), pGSK-3β (Ser9), HSTF-1 and the cleaved form of caspase-3. For evaluation of cytosolic cytochrome *c* levels, the washed cells were suspended in sucrose-supplemented cell extract buffer (SCEB, 300 mM sucrose, 10 mM HEPES at pH 7.4, 50 mM KCl, 5 mM EGTA, 5 mM $MgCl_2$, 1 mM DTT, 10 μM cytochalasin B, 1 mM PMSF), incubated on ice for 30 min, and then homogenized by 50 strokes with an ice-cold Dounce homogenizer. Unbroken cells and nuclei were pelleted by centrifugation at 2000 × *g* for 10 min and discarded. Mitochondria were collected from the resulting supernatant by further centrifugation at 13,000 × *g* for