The Journal of Applied Pharmacology, 13, 256-262(2005)

6-Hydroxydopamine-induced Adaptive Increase in GSH Is Dependent on Reactive Oxygen Species and Ca^{2+} but not on Extracellular Signal-regulated Kinase in SK-N-SH Human Neuroblastoma Cells

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(Received December 6, 2005; Accepted December 22, 2005)

Abstract – We examined the signaling molecules involved in the 6-hydroxydopamine (6-OHDA)-induced neuronal cell death and increase in cellular glutathione (GSH) level in SK-N-SH cells. The 6-OH-DA-induced cell death was significantly prevented by the pretreatment with *N*-acetylcysteine (NAC), a thiol antioxidant, and BAPTA, an intracellular Ca^{2+} chelator. Although 6-OHDA induced ERK phosphorylation, the pretreatment with PD98059, an ERK inhibitor, did not block 6-OHDA-induced cell death. In addition, the 6-OHDA-induced activation of caspase-3, a key signal for apoptosis, was blocked by the pretreatment with NAC and BAPTA. While the level of reactive oxygen species (ROS) was significantly increased in the 6-OHDA-treated cells, the cellular GSH level was not altered for the first 6-hr exposure to 6-OHDA, but after then, the level was significantly increased, which was also blocked by the pretreatment with NAC and BAPTA, but not by PD98059. Depletion of GSH by pretreating the cells with DL-buthionine-(S,R)-sulfoximine (BSO), a glutathione synthesis inhibitor, rather significantly potentiated the 6-OHDA-induced death. In contrast to the pretreatment with NAC, 6-OHDA-induced cell death was not prevented by the post-treatment with NAC 30 min after 6-OHDA treatment. The results indicate that the GSH level which is increased adaptively by the 6-OHDA-induced ROS and intracellular Ca^{2+} is not enough to overcome the death signal mediated through ROS-Ca^{2+}-caspase pathway.

Keywords □ 6-hydroxydopamine, glutathione, *N*-acetylcysteine, BAPTA, Reactive oxygen species, intracellular Ca^{2+}, ERK, caspase-3

Parkinson's disease (PD) is a widespread neurodegenerative disorder that is characterized by the degeneration of dopaminergic neurons in the substantia nigra pars compacta. The mechanism by which these neurons degenerate is still unknown. There has been growing interest in the cellular and molecular mechanisms underlying the degeneration of dopaminergic neurons, in particular, on the dysfunction of complex I of the mitochondrial respiratory chain (Hattori *et al.*, 1991), depletion of glutathione (GSH) (Bharath *et al.*, 2002) and oxidative stress (Floor *et al.*, 1998; Lotharius *et al.*, 2000; Sherer *et al.*, 2002).

6-Hydroxydopamine (6-OHDA) is a selective catecholaminergic neurotoxin. Since the concentration of 6-OHDA has been found to be increased in the brain and urine of Parkinson's patients (Curtius et al., 1974; Andrew *et al.*, 1993), 6-OHDA has been suggested to play a role in the pathogenesis of PD, and has been widely used to produce PD models *in vitro* and *in vivo* (Sauer *et al.*, 1994; Przedborski *et al.*, 1995). Although the precise mechanism of 6-OHDA-induced cytotoxicity has not yet been clarified, it has been suggested that oxidative stress is an important mediator of the neurotoxicity because 6-OHDA is oxidized rapidly by molecular oxygen to form superoxide anion and hydrogen peroxide (Izumi *et al.*, 2005). Reduced glutathione (GSH) and its precursor *N*-acetylcysteine (NAC) that have been recognized as highly effective antioxidants against ROS, protected against neuronal degeneration in 6-OHDA-injected rat striatum (Froissard *et al.*, 1997; Soto-Otero *et al.*, 2000). Although NAC blocks 6-OHDA-induced cell death, it has been shown that cellular GSH level was rather adaptively increased to long term exposure of 6-OHDA.

In addition to the oxidative stress, it has been shown that 6-OHDA induces Ca^{2+} release from mitochondria (Reichman *et al.*, 1994). Even though ROS and intracellular Ca^{2+} have been implicated to play a role n the 6-OHDA-induced cell death, the relationship between such signaling molecules and alterations

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in intracellular GSH level in the 6-OHDA-induced cell death has not yet been clarified.

In the present study, we investigated the possible signaling molecules and their relationships involved in the 6-OHDA-induced neuronal apoptosis and increase in cellular glutathione (GSH) level using SK-N-SH human dopaminergic neuroblastoma cells.

MATERIALS AND METHODS

Cell line and cell culture

The SK-N-SH human neuroblastoma cell line was purchased from American Type Culture Collection (Rockville, MA, USA) and were grown at 37°C in a humidified incubator under 5% CO_2/95% air in Eagle's minimum essential medium (MEM) supplemented with 10% FBS, 1 mM sodium pyruvate, 200 IU/ml penicilline and 200 μg/ml of streptomycin. The culture medium was replaced every other day. The cells were cultured in 96-well plates for cell viability and in 100 mm-diameter dishes for GSH content and enzyme activity assays.

Treatments with 6-OHDA

6-OHDA was dissolved in the culture medium including 0.1 % ascorbic acid. Other inhibitors were co-treated or preincubated 4 hrs or 30 mins prior to the addition of 6-OHDA.

MTT assay

Cell viability was measured by the 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) assay, which is based on the conversion of MTT to formazan crystals by mitochondrial dehydrogenases. Cells were plated at a density of 2×10^4 cells/100 ml medium in a 96-well plate and treated with 6-OHDA for given time. The medium was incubated with 10 μl of 5 mg/ml of MTT solution for 4 hrs at 37°C. Culture medium was removed, and 200 μl of DMSO was added to each well to dissolve formazan. Absorbance was measured at 540 nm using a microplate reader (Molecular Devices, Versa MAX Sunnyvale, CA, USA). The cell viability was expressed as a percent of the control culture.

Determination of apoptosis

Cells were plated at a density of 100,000 cells per well in 6-well plates and were treated with 6-OHDA for up to 24 hrs. For flow cytometry analysis, cells were collected and washed twice with phosphate buffered saline (PBS) (pH 7.4). After fixing in 80% ethanol for 30 mins, cells were washed twice, and resuspended in PBS (pH 7.4) containing 50 μg/ml PI and 25 μg/ml ribonuclease A for DNA staining. The cells were then analyzed using a FACS Calibur flow cytometer (Becton Dickson, CA, USA). At least 20,000 events were evaluated. All histograms were analyzed using Cell Quest (Becton Dickson, CA, USA) to determine percentage of nuclei with hypodiploid content indicative of apoptosis (Hockenbery *et al.*, 1990).

Measurement of caspase-3 activity

Caspase activity was measured by using an ApoAlert caspase colorimetric assay kit (BD Biosciences, San Jose, CA, USA). Briefly, cell lysates were mixed with dithiothreitol (DTT) (10 mM)-rich reaction buffer containing 50 μM DEVD-pNA, a caspase-3 substrate, and incubated for 1 hr at 37°C. Enzyme-catalyzed release of pNA was monitored using a microplate reader at 405 nm.

Western blot analysis

Cells were lysed in freshly prepared extraction buffer [10 mM Tris-HCl (pH 7.6), 0.1% Nonidet P-40, 150 mM NaCl, 10 mM EDTA, 1 mM phenylmethylsulfonyl floride, and protease inhibitor cocktail (Sigma-Aldrich, MO, USA)] for 30 mins at 4°C. Protein detection was performed using a Bradford reagent (Bio-Rad, CA, USA). Lysates were centrifuged at 20,000 *g* for 10 min at 4°C, and supernatant proteins were separated on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a Hybond ECL nitrocellulose membrane (Amersham Life Science, Buckinghamshire, England). The membrane was blocked with 5% skim milk in Tween-20 containing Tris buffered saline (TTBS) (20 mM Tris–HCl (pH 7.6), 150 mM NaCl, 0.05% Tween-20) and incubated with primary anti-human pERK, (Santa Cruz Biotechnology, CA, USA) and ERK (Cell Signaling Technology, Beverly, MA, USA) antibodies in TTBS containing 3% skim milk. After incubation with horseradish peroxidase-conjugated anti-IgG antibody (Santa Cruz Biotechnology, CA, USA), immunodetected proteins were visualized by using an enhanced chemiluminescence assay kit (Amersham Biosciences, Buckinghamshire, England). The density of the protein bands was measured by using Image Analyzing System (UVP, Upland, USA).

Measurement of ROS generation (Nitroblue tetrazolium reduction assay)

Intracellular superoxide generation was measured by using conversion of nitroblue tetrazolium (NBT) to formazan. NBT was added to the medium of cells to a final concentration of 1 mg/ml. After AA treatment, cells were lysed and formazan was

dissolved with 2 M KOH and 1.4 volume of DMSO. The absorbance was read spectrophotometrically at 654 nm.

Measurements of total glutathione concentration

The contents of total glutathione (GSH+GSSG) were determined using a total glutathione quantification kit (Cayman Chemical Company, USA). In this assay, the sulfhydryl group of GSH reacts with DTNB (5,5-dithio-bis-2-nitrobenzoic acid, Ellman's reagent) and produces a yellow colored 5-thio-2-nitrobenzoic acid (TNB). The mixed disulfide, GSTNB (between GSH and TNB) that is concomitantly produced, is reduced by glutathione reductase to recycle the GSH and produced more TNB. The rate of TNB production is directly proportional to this recycling reaction which is in turn directly proportional to the concentration of GSH in the sample. Measurement of the absorbance of TNB at 405 or 414 nm provides an accurate estimation of GSH in the sample. In brief, cells were washed in PBS, lysed in 50 mM MES buffer [0.2 M 2-(N-morpholino) ethanesulphonic acid, 0.05 M phosphate, and 1 mM EDTA, pH 6.0], sonicated, and centrifuged at 10,000 × g for 15 mins at 4°C. The supernatant was deproteinated by adding equal volume of the MPA reagent (10% of metaphosphoric acid), and subsequently centrifuged at 5,000 × g for 5 mins. Supernatants were stored at -80°C until measurement. Incubation was initiated by mixing a reaction solution cocktail containing glucose-6-phosphate, NADPH, glutathione reductase, glucose-6-phosphate dehydrogenase and DTNB in a 96-well plate and shaking in the dark. The absorbance was measured at 405 nm. Total glutathione concentration was calculated from standard curves.

Data analysis

The data were expressed as the mean±standard error of the mean (SEM) and analyzed using one-way analysis of variance (ANOVA) and Student–Newman–Keul's test for individual comparisons. *P* values less than 0.05 were considered statistically significant.

RESULTS

6-OHDA-induced apoptosis is dependent on the level of ROS and Ca^{2+} in SK-N-SH cells

The treatment of SK-N-SH cells with 6-OHDA for 48 hrs

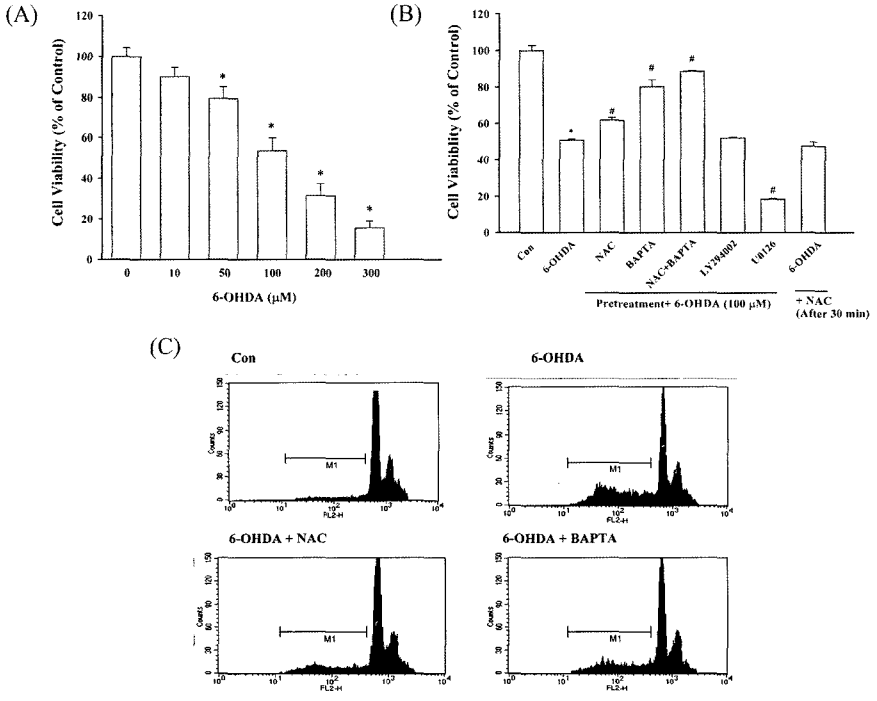

Fig. 1. The 6-OHDA-induced cell death is blocked by the pretreatment with NAC and BAPTA. In the experiments of (A), cells were treated for 48 hrs with or without each concentration of 6-OHDA, and the cell viability was measured by MTT method. In the experiments of (B), cells were pretreated with BAPTA (2 μM) for 4 hr, and NAC (1 mM), LY294002 (10 μM) and U0126 (20 μM) for 30 mins prior to 6-OHDA treatment. In the experiments of (C), the drug-pretreated cells were incubated in the presence of 6-OHDA (100 μM) for 24 hrs. Cells were then stained with Annexin-V-FITC and analyzed by flow cytometry. Data points represent the mean values of four replications with bars indicating SEM. $^{*}P<0.05$, compared to 6-OHDA untreated control. $^{\#}P<0.05$ compared to 6-OHDA-treated group.

significantly decreased the cell viability at concentrations between 10 and 300 μM, as shown in Fig. 1A. To determine which signal is involved in the decrease in cell viability of 6-OHDA-treated cells, we pretreated the cells with NAC, a thiol antioxidant, BAPTA, an intracellular Ca^{2+} chelator, U0126, a mitogen-activated protein kinase (MAPK) pathway specific inhibitor, and LY294002, an inhibitor of phosphatidylinositol 3-kinase. As shown in Fig 1B, the pretreatment with NAC and BAPTA significantly suppressed the 6-OHDA-induced decrease in cell viability, whereas U0126 and LY294002 did not. In addition, the combined treatment with both NAC and BAPTA synergistically suppressed the 6-OHDA-induced cell death. Furthermore, flow cytometry analysis using PI-staining method showed that 6-OHDA induced accumulation of the cells at sub-G_0/G_1 phase, a characteristic of apoptotic cells, which was also prevented with the pretreatment with NAC and BAPTA, as depicted in Fig. 1C. In order to confirm these signals are involved in the 6-OHDA-induced apoptosis, we measured the changes in the caspase-3 activity. The 6-OHDA-induced activation of caspase-3 (Fig. 2A) was also significantly blocked by the pretreatment with NAC and BAPTA (Fig. 2B).

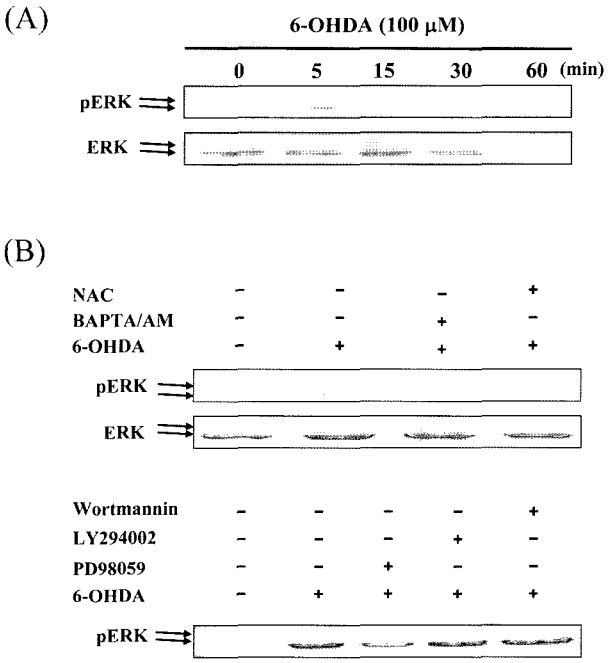

Fig. 3. The effects of NAC and various Ca^{2+} inhibitors on 6-OHDA-induced ERK activation in SK-N-SH cells. In the experiments of (A), cells were treated with 6-OHDA for the designated time. In the experiments of (B), BAPTA/AM (2 μM) was pretreated for 4 hrs, and NAC (100 mM), wortmannin (100 μM), LY294002 (10 mM) and PD98059 (10 μM) were pretreated 30 mins prior to 6-OHDA treatment. The equal amounts of protein (50 μg) were subjected to immunoblot analysis using antibody against the phosphorylated form of ERK1/2. The blots were then stripped and reprobed with antibody against total ERK1/2.

ROS-dependent ERK activation is not involved in the 6-OHDA-induced cell death

Since it has been reported that extracellular signal-regulated kinases (ERK) plays a pivotal role in 6-OHDA-induced neurotoxicity (Kulich and Chu, 2001; 2003), we also examined the role of ERK in the 6-OHDA-induced cell death. In fact, 6-OHDA induced phosphorylation of ERK at 5 min and up to 1 hr, as depicted in the Fig. 3A, which was significantly blocked by NAC. However, 6-OHDA induced phosphorylation of ERK that was inhibitable by PD98059, an ERK inhibitor, was not

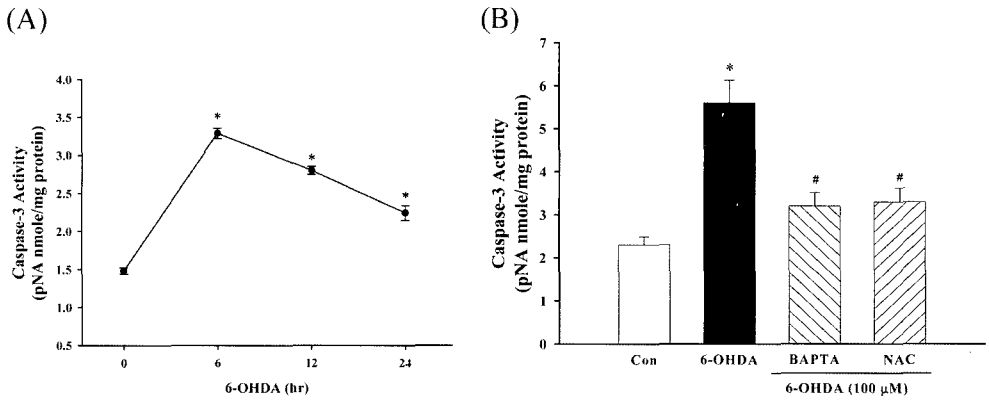

Fig. 2. Activation of caspase-3 is blocked by NAC and BAPTA in 6-OHDA-treated SK-N-SH cells. Cells were either treated with 6-OHDA for each designated time (A) or pretreated with NAC (100 mM) for 30 min and BAPTA/AM (2 μM) for 4 hrs prior to 100 μM of 6-OHDA (B). Caspase activity was measured by using an ApoAlert caspase colorimetric assay kit. *P<0.05 compared to control. #P<0.05 compared to 6-OHDA alone.

prevented by the pretreatment with BAPTA, TMB-8, an intracellular Ca^{2+} release blocker, or PI3-Kinase inhibitors wortmannin and LY294002, as shown in Fig. 3B.

6-OHDA-induced adaptive increase in GSH level is preventable by the treatment with NAC and BAPTA, but not with PD98059

Since we also found that 6-OHDA increased the level of intracellular ROS (Fig. 4), next, we examined the effect of 6-OHDA on endogenous glutathione levels in SK-N-SH cells.

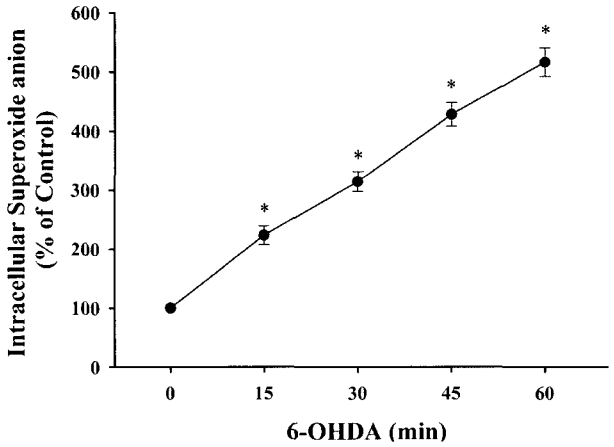

Fig. 4. 6-OHDA increases cellular level of ROS. The basal level of ROS as a function of time was measured by NBT method, and expressed as fold increase compared to the control intensity. Data points represent the mean values of four replications with bars indicating SEM. *P<0.05, compared to 6-OHDA untreated control.

The contents of total glutathione (GSH + GSSG) measured using a total glutathione quantification kit was not changed up to 6 hrs after treatment with 100 μM 6-OHDA (Fig 5A), but after 12 hrs, the level was significantly increased more than 1.6-fold over the control, which was prevented by the pretreatment with NAC and BAPTA, but not by PD98059 (Fig. 5B). However, as shown in Fig. 6, the depletion of GSH by pretreating the cells with DL-buthionine-(S,R)-sulfoximine (BSO), a glutathione synthesis inhibitor, rather potentiated the cytotoxicity of 6-OHDA in the cells. In contrast to the pretreatment, the cell death by 6-OHDA was not suppressed when NAC was added to the 6-OHDA-treated cells 30 mins later, as depicted in Fig. 1B.

DISCUSSION

Although the precise mechanism of 6-OHDA-induced cytotoxicity has not been clarified, it is well known that 6-OHDA is rapidly oxidized by molecular oxygen to form superoxide anion and hydrogen peroxide. The ROS and the oxidized products including p-quinone and aminochrome generated during 6-OHDA oxidation are focused to be the primary causes of 6-OHDA-induced cytotoxicity (Glinka *et al.*, 1995; Shimizu *et al.*, 2002). In the present study, we also found that 6-OHDA increases ROS level, which contribute to the 6-OHDA-induced cell death since NAC, a thiol antioxidant blocked the cell death (Fig. 1B and 1C). In addition, the 6-OHDA-induced cell death was prevented by the intracellular Ca^{2+} chelator BAPTA, of which result is consistent and even more supportive to the pre-

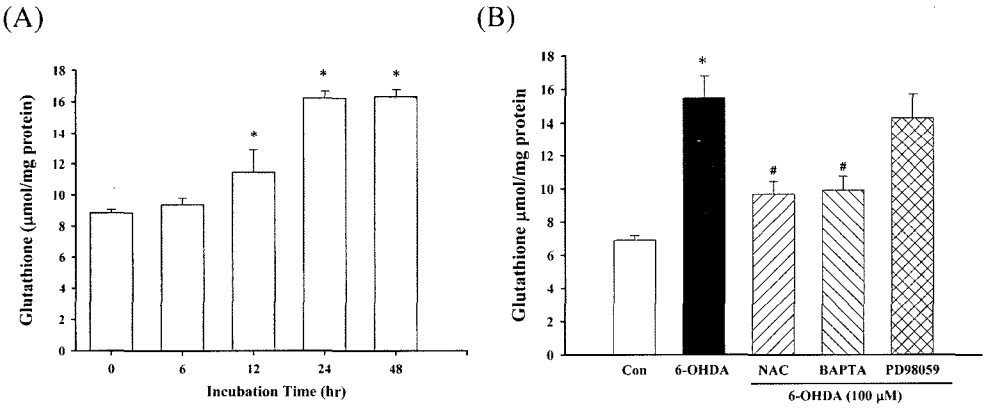

Fig. 5. The effects of NAC and BAPTA on the 6-OHDA-induced glutathione level. In the experiments of (A), cells were exposed to 100 μM of 6-OHDA for different time. The total GSH content was determined using a GSH quantification kit. In the experiments of (B), cells were pretreated with BAPTA (2 μM) and NAC (1 mM) prior to the treatment with 6-OHDA for 24 hrs. Data points present the mean values of four replications with bars indicating SEM. *P<0.05, compared to 6-OHDA untreated control, #P<0.05 compared to 6-OHDA-treated group.

vious reports that high levels of ROS could induce secondary excitotoxicity by raising intracellular Ca^{2+} concentration, and that 6-OHDA induces Ca^{2+} release from mitochondria (Reichman *et al.*, 1994). More importantly, our results indicate that the two signals, ROS and Ca^{2+}, may work in a parallel mode since the cotreatment with both NAC and BAPTA synergistically suppressed the 6-OHDA-induced cell death, and only one signal among the two is related to the activation of ERK.

It has been known that ERK activation plays a pivotal role in regulating neuronal decisions to live or die in response to stressors (Xia *et al.*, 1995). Although the level of phospho-ERK has been found to be increased in the neurons of substantia nigra of Parkinson's disease patients, the effects of ERK inhibition are rather contradictory, representing both a detrimental role (Otani et al., 2002) and a functional recovery-promoting role (Dash *et al.*, 2002). Such contradictory results may be ascribed to the nature and severity of injury, drug dosing regimens and the cell types. Although our results showing that the ERK inhibition did not block 6-OHDA-induced cell death in SK-N-SH cells is also contradictory to the previous reports that sustained ERK activation is also been related to the 6-OHDA-induced neurotoxicity in B65 cells (Kulich and Chu, 2001; 2003), in the present study, we found that ERK is not activated by Ca^{2+} signal, but by ROS (Fig. 3).

In addition to the oxidative stress, it has been reported that 6-OHDA directly and reversibly inhibits complex I activity of the mitochondrial electron transport chain (Glinka *et al.*, 1996), which is known to contribute to the decrease in GSH level (Ambani *et al.*, 1975; Kish *et al.*, 1985). However, it has been documented that 6-OHDA or its metabolites up-regulate GSH level (Shimizu *et al.*, 2002; Emdadul *et al.*, 2003; Tirmenstein *et al.*, 2005). Similar to the previous above reports, our results also showed that 6-OHDA altered cellular GSH level only after several hours of 6-OHDA exposure (Fig. 5A). Furthermore, although BSO itself, a GSH synthesis inhibitor, did not induce cell death, co-treatment with BSO enhanced the 6-OHDA-induced cell death, indicating that GSH-depleted cells were more susceptible to 6-OHDA, and that the GSH increase in the 6-OHDA-treated cells might be a compensatory process against 6-OHDA-induced toxicity. More importantly, pretreatment with NAC and BAPTA, which showed a potent protective effect on 6-OHDA toxicity, attenuated GSH up-regulation. In our study, NAC and BAPTA not only blocked 6-OHDA-induced apoptotic cell death and activation of caspase-3, but also prevented the increase in GSH level by 6-OHDA, suggesting that ROS and Ca^{2+} may act upstream signals leading to the caspase-3 activation and up-regulation of intracellular GSH.

In summary, these results indicate that the GSH level which is increased adaptively by the 6-OHDA-induced ROS and intracellular Ca^{2+} is not enough to overcome the death signal mediated through ROS- Ca^{2+}-caspase pathway.

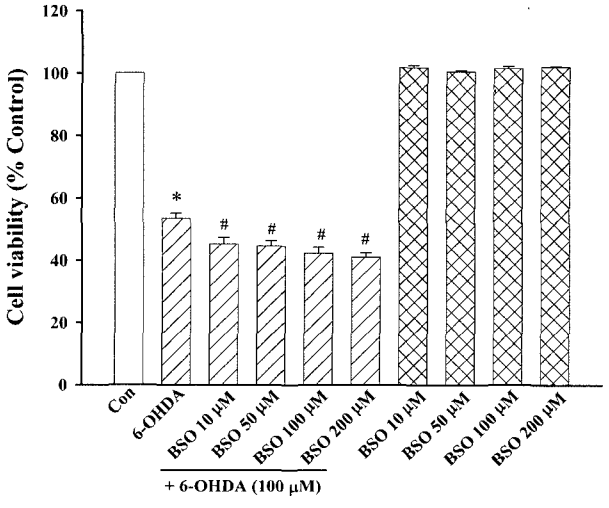

Fig. 6. BSO, a glutathione synthesis inhibitor, potentiates the 6-OHDA-induced cell death. BSO was co-treated with 6-OHDA for 48 hr, and cell viability was analyzed by MTT assay. **P*<0.05 compared to control. #*P*<0.05 compared to 6-OHDA alone.

ACKNOWLEGMENTS

This work was supported by Korea Research Foundation Grant (KRF-2003-041-E00329).

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