Stimulus-dependent neuronal cell responses in SH-SY5Y neuroblastoma cells

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Abstract. The aim of the present study was to elucidate the intracellular mechanisms that cause neuronal cell death following exposure to excitatory neurotransmitter-induced neurotoxicity, neurotoxins and oxidative stress. Human SH-SY5Y neuroblastoma cells were exposed to various stimuli, including glutamate, 6-hydroxydopamine (6-OHDA), and glucose oxidase, and cell viability was determined by MTT assay. Early apoptosis and necrosis were examined by Annexin V/propidium iodide double staining and flow cytometric analysis. Intracellular calcium ion concentration and mitochondrial membrane potential were assessed by Fluo-3a and JC-1 staining, respectively. In addition, protein expression of receptor-interacting protein (RIP) kinase 1 and RIP kinase 3 were evaluated by western blotting. Glutamate, 6-OHDA and glucose oxidase treatment decreased cell viability. Glutamate induced apoptosis and necrosis, whereas, 6-OHDA induced cell necrosis and glucose oxidase induced apoptosis. Furthermore, glutamate, 6-OHDA or glucose oxidase treatment significantly increased intracellular calcium concentrations ($P<0.05$). The effect of glutamate on mitochondrial membrane potential varied with high and low concentrations, whereas 6-OHDA and glucose oxidase significantly increased the mitochondrial membrane potential in the SH-SY5Y cells ($P<0.05$). Glutamate significantly upregulated expression levels of RIP kinase 1 ($P<0.05$), but not RIP kinase 3. These findings demonstrate that the response of SH-SY5Y cells varies with the stimuli. Furthermore, RIP kinase 1 may specifically regulate programmed necrosis in glutamate-mediated excitatory toxicity, but not in cell damage induced by either 6-OHDA or glucose oxidase.

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Introduction

Neuronal cell death is a normal physiological process during nervous system development. However, it is also a pathological process in brain and spinal cord disease and injury (1). Abnormal neuronal cell death in the central nervous system (CNS) is also associated with acute neurological injury, such as cerebral ischemia and trauma, as well as chronic neurodegenerative disorders, such as Alzheimer's disease and Parkinson's disease (PD). Excitatory neurotransmitter-induced neurotoxicity, neurotoxins, and oxidative stress are commonly used to induce neuronal cell death in *in vivo* and *in vitro* models (2-4).

It has been proposed that apoptosis and necrosis are involved in neuronal cell death, which is caused by excitatory neurotransmitter-induced neurotoxicity, neurotoxins and oxidative stress (5). Apoptosis is a programmed cell death pathway, which is regulated by an evolutionarily conserved cellular pathway that involves activation of the caspase cascade (6). Programmed necrosis (also called necroptosis in cell cultures) is an essential process of animal development, but also leads to human diseases (7). Notably, cell death following neonatal brain injury is caused by programmed necrosis (8). This indicates the critical role of programmed necrosis in neuronal cell death following injury. The mechanism of programmed necrosis has not been fully elucidated. Receptor-interacting protein (RIP) kinase 1 and RIP kinase 3, are hypothesized to be the key signaling molecules in programmed necrosis, and the two may be regulated by caspases and ubiquitination (9,10). The mechanisms by which neuronal cells die in response to excitatory neurotransmitter-induced neurotoxicity, neurotoxins and oxidative stress have not been determined.

In the present study, the mechanism by which human SH-SY5Y neuroblastoma cells die in response to glutamate-induced neurotoxicity, 6-hydroxydopamine (6-OHDA)-induced neurodegenerative toxicity and glucose oxidase-induced oxidative stress was investigated. The findings may provide the basis for understanding the mechanism by which neuronal cells die in response to these types of stimuli.

Materials and methods

Reagents. Glutamate, 6-OHDA, glucose oxidase and dithiothreitol were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from GE Healthcare Life Sciences (Logan, UT, USA). BD™ MitoScreen (JC-1) and an Annexin V/propidium iodide (PI) kit were purchased from BD Biosciences (San Jose, CA, USA). A calcium fluorescent probe (Fluo-3 AM) was purchased from Beyotime Institute of Biotechnology (Wuhan, China). Mouse anti-human anti-RIP kinase 1 (ab56815) and rabbit anti-human anti-RIP kinase 3 (ab180535) primary antibodies were obtained from Abcam (Cambridge, UK). Alexa Fluor 680-conjugated goat anti-mouse immunoglobulin G (IgG) (A28183) and Alexa Fluor 790-conjugated goat anti-mouse IgG (A28182) secondary antibodies were purchased from Thermo Fisher Scientific, Inc. (Waltham, MA, USA), and tetramethylethylenediamine was purchased from Sangon Biotech Co., Ltd. (Shanghai, China). Chemical reagents used for western blot analysis, including glycine, bis-acrylamide, and acrylamide were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), and ammonium persulfate was obtained from Sangon Biotech Co., Ltd.

Cell culture. Human SH-SY5Y neuroblastoma cells were obtained from the China Center for Type Cultural Collection (Wuhan University, Wuhan, China). The cells were cultured in DMEM culture medium supplemented with 10% FBS and maintained at 37˚C in a 5% CO_2 humidified atmosphere. Upon reaching 80% confluence, the cells were digested with 0.25% trypsin for 3 min until the majority of the cells detached. The cells were subsequently resuspended with DMEM culture medium containing 10% FBS. Following centrifuging at 97 x g for 10 min, the supernatant was removed and cells were resuspended with fresh culture medium. The cell density was adjusted to $5x10^5$ cells per well for each 6-well plate or $1x10^4$ cells per well for each 96-well plate. Subculturing was performed every three or four days, upon the cells reaching 80% confluence.

Glutamate neurotoxicity model. One day after initial seeding, cells were exposed to different concentrations of glutamate (10, 15, 20, 25 or 50 mM) diluted in serum-free DMEM culture medium. Non-treated cells served as a negative control. The culture medium was removed following an exposure time of 2, 4, 6 or 8 h. The cells were washed twice with phosphate-buffered saline (PBS) or distilled water and collected for subsequent experiments.

6-OHDA-induced neurodegenerative toxicity model. One day after initial seeding, cells were exposed to varying concentrations of 6-OHDA (0.05, 0.25, 0.5, 1 or 2 mM) dissolved in serum-free DMEM culture medium. Non-treated cells served as a negative control. The culture medium was removed following an exposure time of 4, 8 or 12 h. The cells were washed twice with PBS or distilled water and collected for subsequent experiments.

Glucose oxidase oxidative stress model. One day after initial seeding, cells were exposed to different concentrations of glucose oxidase (0.005, 0.05, 0.5, 5 or 20 mg/ml) dissolved in serum-free DMEM culture medium. Non-treated cells served as a negative control. The culture medium was removed following an exposure time of 10, 20, 40 or 60 min. The cells were washed twice with PBS or distilled water and collected for subsequent experiments.

Determination of cell viability. Cells were seeded into 96-well plates ($1x10^4$ cells per well). Subsequent to the appropriate incubation time with the indicated compound concentrations, 100 μl MTT (0.5 mg/ml; Sangon Biotech Co., Ltd.) was added to each well. The cells were incubated for 4 h at 37˚C, the medium was removed and 150 μl dimethyl sulfoxide (Sangon Biotech Co., Ltd.) was added to each well to resuspend the MTT metabolic product. The absorbance of the dissolved formazan was measured at 490 nm with a microplate reader (Model 550; Bio-Rad Laboratories, Inc., Hercules, CA, USA). Data were calculated from three independent experiments.

Evaluation of cell apoptosis and necrosis. Cells were seeded into 6-well plates ($5x10^5$ cells per well). Following the appropriate incubation period, the culture medium was removed and cells were washed with PBS. Cells were collected by centrifuging at 67 x g for 5 min. Following PBS washing, $3x10^4$ cells were resuspended with the staining solution containing 5 μl PI and 5 μl Annexin V. The samples were examined by flow cytometry (BD FACSCalibur™; BD Biosciences) after a 30-min incubation in the dark. Annexin V-positive/PI-negative cells were identified as early apoptotic cells, and Annexin V-positive/PI-positive cells were identified as late apoptotic and necrotic cells.

Assessment of intracellular calcium ion concentration. Cells were seeded into 6-well plates ($5x10^5$ cells per well). Subsequent to the appropriate incubation period, the culture medium was removed and cells were washed twice with distilled water. Cells were probed with 500 μl Fluo-3a solution for 1 h at 37˚C. Following incubation, the Fluo-3a solution was removed and 2 ml distilled water was added to resuspend the cells. Cells were collected by centrifuging at 67 x g for 5 min and resuspended with distilled water. The samples were examined by flow cytometry, using the BD FACSCalibur™.

Analysis of mitochondrial membrane potential. Cells were seeded into 6-well plates ($5x10^5$ cells per well). Following the appropriate incubation period, the culture medium was removed and cells were washed twice with PBS. Cells were probed with 500 μl 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolocarbocyanine iodide (JC-1) staining solution for 30 min at 37˚C. Following incubation, the JC-1 solution was removed and 200 μl PBS was added to resuspend the cells. The samples were then examined by flow cytometry, using the BD FACSCalibur™.

Western blot analysis. Cells were seeded into 6-well plates ($5x10^5$ cells per well). Following the appropriate incubation period, the culture medium was removed and cells were washed twice with PBS. Total protein was extracted from cells using radioimmunoprecipitation analysis lysis buffer containing 1 mM phenylmethylsulfonyl fluoride (both Sangon Biotech Co., Ltd.) and maintained for 30 min on ice. After centrifuging at 13,160 x g for 4 min at 4˚C, the supernatant was carefully collected and stored at -20˚C until use. Protein concentrations

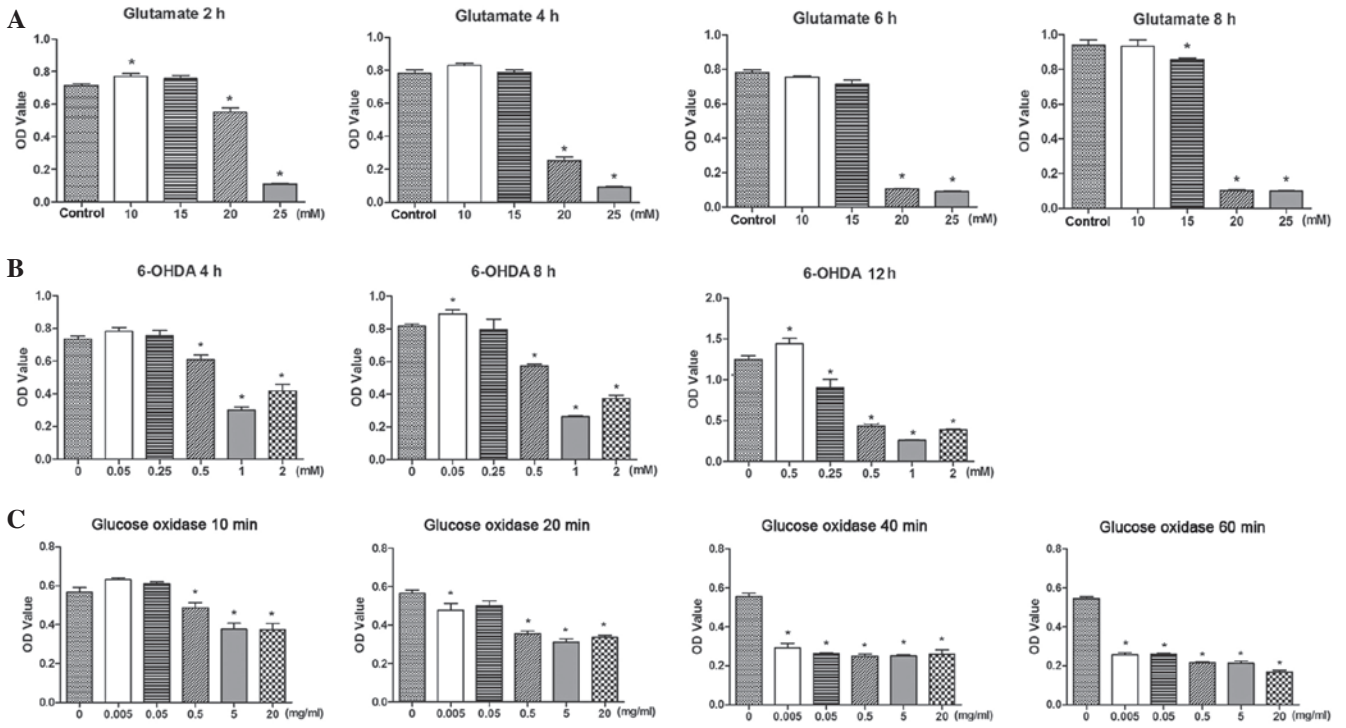

Figure 1. Decreased viability of SH-SY5Y cells following exposure to various stimuli. Cell viability was determined by the MTT assay. (A) Cells were treated with 0, 10, 15, 20 or 25 mM glutamate for 2, 4, 6 or 8 h. (B) Cells were treated with 0, 0.05, 0.25, 0.5, 1 or 2 mM 6-OHDA for 4, 8 or 12 h. (C) Cells were treated with 0, 0.005, 0.05, 0.5, 5 or 20 mg/ml glucose oxidase for 10, 20, 40 or 60 min. Data are presented as the mean ± standard deviation of three independent experiments. *P<0.05 vs. the control (0). OD, optical density; 6-OHDA, 6-hydroxydopamine.

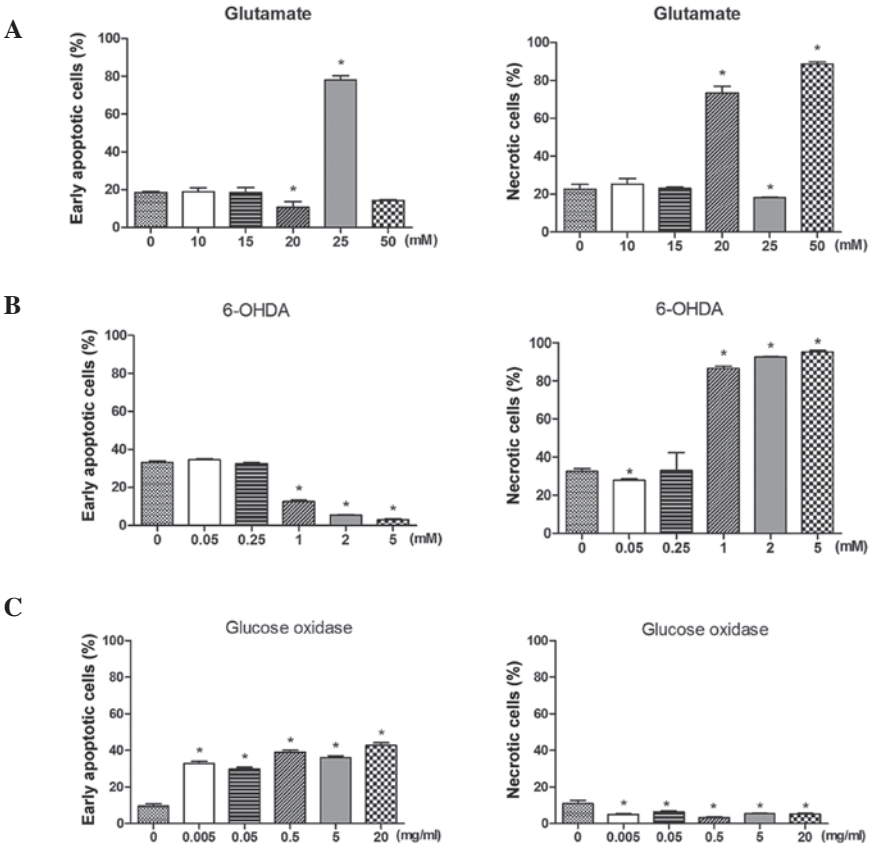

Figure 2. Differential induction of apoptosis and necrosis of SH-SY5Y cells induced by various stimuli. Cells were double stained with Annexin V and propidium iodide. The percentages of early apoptotic or necrotic cells were determined by flow cytometric analysis. (A) Cells were treated with 0, 10, 15, 20, 25 or 50 mM glutamate for 1 h. (B) Cells were treated with 0, 0.05, 0.25, 1, 2 or 5 mM 6-OHDA for 1 h. (C) Cells were treated with 0, 0.005, 0.05, 0.5, 5 or 20 mg/ml glucose oxidase for 20 min. Data are presented as the mean ± standard deviation of three independent experiments. *P<0.05 vs. the control (0). 6-OHDA, 6-hydroxydopamine.

(1 μg/μl) were measured using the BCA Protein Assay kit (Beyotime Institute of Biotechnology). Equal quantities of protein extracts (50 ng) were then separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes (Sangon Biotech Co., Ltd.). The membranes were blocked with Tris-buffered saline plus Tween-20 (TBS-T; 0.1% Tween-20) containing 5% w/v non-fat dry milk (Sangon Biotech Co., Ltd.), then incubated with the primary antibodies (dilution, 1:1,000) at room temperature for 2 h. After washing with TBS-T, the membranes were incubated with secondary antibodies (dilution, 1:1,000) for 1 or 2 h at room temperature. To quantify the protein level, the expression bands of the target proteins were analyzed using the Odyssey Infrared Imaging System (LI-COR Biotechnology, Lincoln, NE, USA). Densitometric values obtained from western blotting were used to conduct statistical analysis and the housekeeping protein, GAPDH served as an internal control.

Statistical analysis. Data were analyzed by SPSS statistical software package, version 5 (SPSS, Inc., Chicago, IL, USA) and all data are presented as the mean ± standard deviation. Statistical significance was determined using the two-way analysis of variance comparison test and $P<0.05$ was considered to indicate a statistically significant difference.

Results

Cell viability is decreased by high doses of glutamate, 6-OHDA and glucose oxidase. The effect of glutamate, 6-OHDA and glucose oxidase on cell viability was examined. A significant reduction of cell viability was detected when cells were exposed to glutamate doses >20 mM (Fig. 1A; $P<0.05$). Cell viability was significantly decreased in cells exposed to concentrations of 6-OHDA >0.5 mM at all time points (Fig. 1B; $P<0.05$). Furthermore, cell viability was significantly reduced following exposure to >5 mg/ml glucose oxidase (Fig. 1C; $P<0.05$). Overall, a prolonged incubation duration or increased concentration decreased cell viability for the three stimuli that were assessed in the SH-SY5Y cells.

Differential induction of apoptosis and necrosis by glutamate, 6-OHDA and glucose oxidase. Apoptosis and necrosis induced by glutamate, 6-OHDA and glucose oxidase was investigated using Annexin V/PI staining followed by flow cytometry. Glutamate treatment induced early apoptosis and necrosis in the SH-SY5Y cells (Fig. 2A). Apoptosis was significantly induced after cells were exposed to 25 mM glutamate for 1 h ($P<0.05$). In addition, exposure to 20 and 50 mM glutamate significantly increased the percentage of necrotic cells ($P<0.05$). The percentage of necrotic cells was significantly increased when compared with untreated cells after 1 h of incubation with 1, 2 or 5 mM 6-OHDA (Fig. 2B; $P<0.05$). By contrast, early apoptosis was significantly induced following glucose oxidase incubation (Fig. 2C; $P<0.05$). These data indicate that glutamate induced apoptosis and necrosis, whereas 6-OHDA only induced necrosis and glucose oxidase only induced early apoptosis.

Intracellular calcium ion concentration is increased following exposure to glutamate, 6-OHDA and glucose oxidase. Cells were stained with Fluo-3a following exposure to glutamate, 6-OHDA or glucose oxidase to determine the resulting intracellular calcium ion level. The intracellular calcium concentration was observed to be dose-dependently elevated following exposure to glutamate (15-25 mM) for 1 h, (Fig. 3A; $P<0.05$ vs. the control). However, no significant difference was identified in the intracellular calcium concentration of cells treated with 10 or 50 mM glutamate when compared with the control cells ($P>0.05$). Furthermore, 6-OHDA (>0.05 mM) and glucose oxidase (>0.05 mg/ml) dose-dependently increased the intracellular calcium concentration (Fig. 3B and C; $P<0.05$). These results indicate that glutamate, 6-OHDA and glucose oxidase exposure increases intracellular calcium ion concentrations. Increased intracellular calcium ion concentrations may result in cell damage.

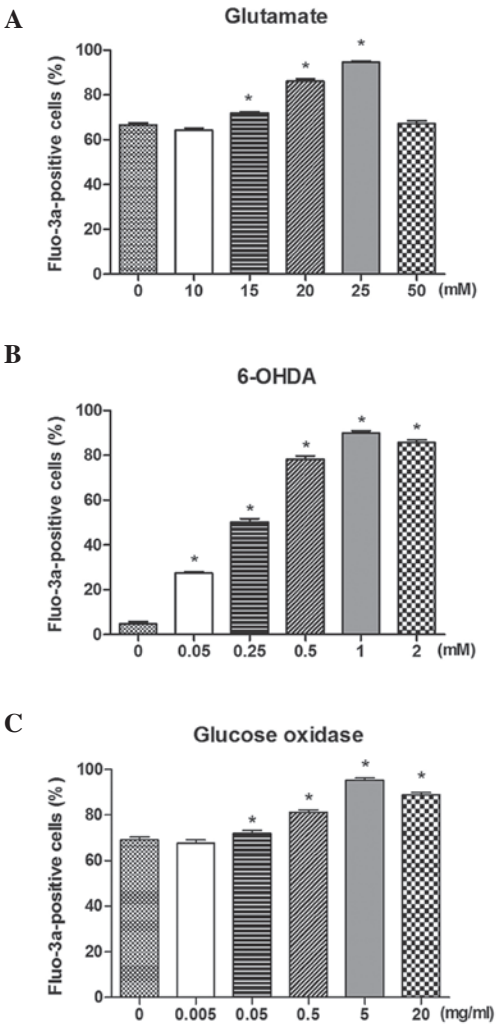

Figure 3. Intracellular calcium ion concentration was increased following exposure to various stimuli. Following each treatment, cells were stained with Fluo-3a and analyzed by flow cytometry. The percentages of Fluo-3a-positive cells are presented. (A) Cells were treated with 0, 10, 15, 20, 25 or 50 mM glutamate for 1 h. (B) Cells were treated with 0, 0.05, 0.25, 0.5, 1 or 2 mM 6-OHDA for 1 h. (C) Cells were treated with 0, 0.005, 0.05, 0.5, 5 or 20 mg/ml glucose oxidase for 20 min. Data are presented as the mean ± standard deviation of three independent experiments. *$P<0.05$ vs. the control (0). 6-OHDA, 6-hydroxydopamine.

Mitochondrial membrane potential increased following glutamate, 6-OHDA and glucose oxidase exposure. The changes in mitochondrial membrane potential of cells exposed to glutamate,

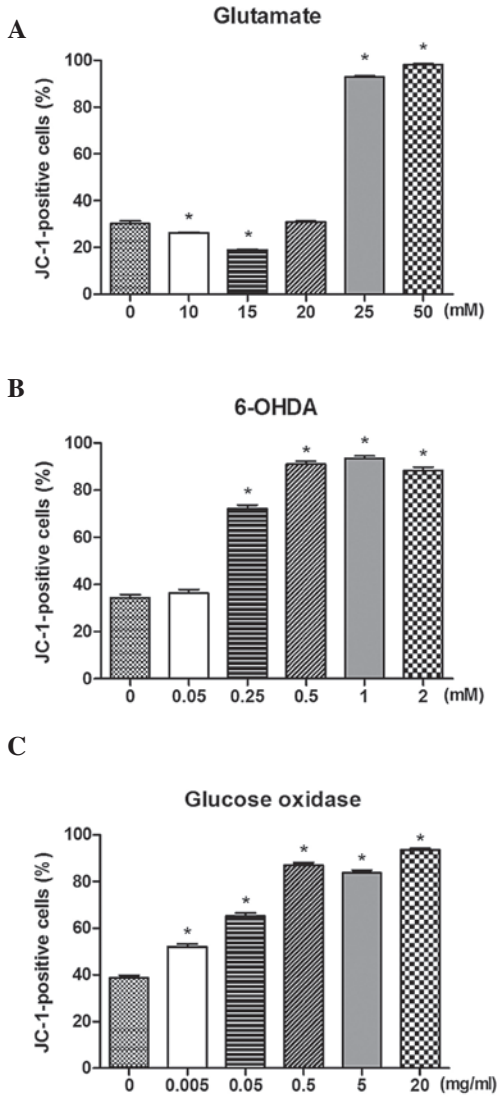

Figure 4. Mitochondrial membrane potential following exposure to various stimuli. Following each treatment, cells were stained with JC-1 and analyzed by flow cytometry. The percentage of JC-1-positive cells are presented. (A) Cells were treated with 0, 10, 15, 20, 25 or 50 mM glutamate for 1 h. (B) Cells were treated with 0, 0.05, 0.25, 0.5, 1 or 2 mM 6-OHDA for 1 h. (C) Cells were treated with 0, 0.005, 0.05, 0.5, 5 or 20 mg/ml glucose oxidase for 20 min. Data are presented as the mean ± standard deviation of three independent experiments. *P<0.05 vs. the control (0). 6-OHDA, 6-hydroxydopamine.

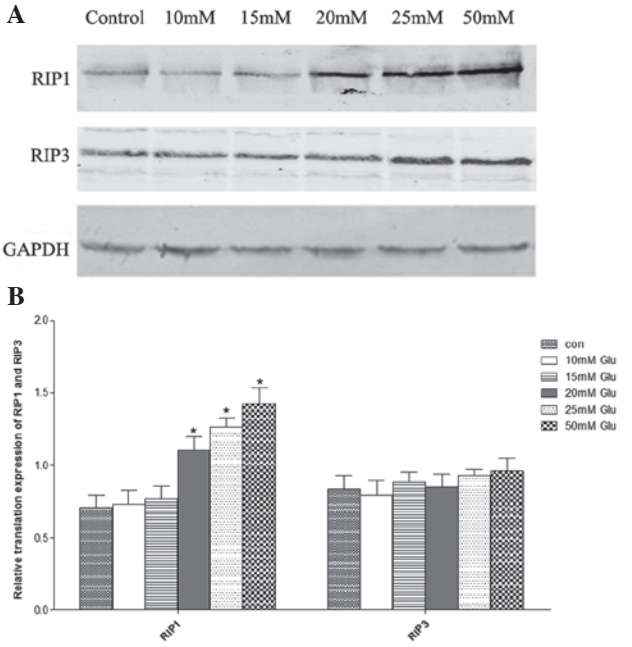

Figure 5. Upregulation of RIP kinase 1 protein expression following glutamate treatment. (A) Cells were treated with 0, 10, 15, 20, 25 or 50 mM glutamate for 2 h. The protein expression of RIP kinase 1 and 3 was evaluated using western blotting. (B) Western blot data quantified by densitometry. Data are presented as the mean ± standard deviation of three independent experiments. *P<0.05 vs. the control (0). RIP, receptor-interacting protein; Glu, glutamate; Con, control.

6-OHDA and glucose oxidase were determined by JC-1 staining. As shown in Fig. 4A, low doses of glutamate (10 or 15 mM) significantly decreased the mitochondrial membrane potential in SH-SY5Y cells (P<0.05), whereas high doses of glutamate (25 or 50 mM) significantly increased the mitochondrial membrane potential in the cells (P<0.05). Concentrations of 6-OHDA that were >0.25 mM and glucose oxidase concentrations that were >0.005 mg/ml significantly increased the mitochondrial membrane potential of the cells (Fig. 4B and C; P<0.05). These findings indicate that the mitochondrial membrane potential response varied depending on the concentration of glutamate (high or low), whereas 6-OHDA and glucose oxidase exposure of any concentration increased the mitochondrial membrane potential in the SH-SY5Y cells. Elevated mitochondrial membrane potential may contribute to neuronal cell death.

RIP kinase 1 expression is induced following glutamate treatment. The expression levels of RIP kinase 1 and RIP kinase 3 (key signaling molecules in necrosis) following exposure to glutamate, 6-OHDA and glucose oxidase were determined by western blotting. The expression of RIP kinase 1 was significantly upregulated in a dose-dependent manner as a result of glutamate treatment (Fig. 5A; P<0.05). However, no significant difference was detected in the expression level of RIP kinase 3 in cells that were treated with glutamate. In addition, 6-OHDA and glucose oxidase treatment did not alter the levels of either RIP kinase 1 or 3 (data not shown). This data indicates that the RIP kinase 1 signaling pathway may be involved in glutamate-induced excitatory toxicity.

Discussion

Neuronal cells may exhibit varying responses depending on the type of stimuli. Stimuli, such as excitatory neurotransmitter-induced neurotoxicity, neurotoxins and oxidative stress may induce neuronal cell death (2-4). In the present study, glutamate, 6-OHDA and glucose oxidase were used to mimic excitatory neurotransmitter-induced neurotoxicity, neurotoxins, and oxidative stress in human SH-SY5Y neuroblastoma cells in order to elucidate the intracellular mechanisms that cause neuronal cell death.

Glutamate is the primary excitatory neurotransmitter in the brain. It is associated with various CNS disorders, such as stroke (11), epilepsy (12) and neurodegenerative diseases (3). In the current study, glutamate suppressed cell viability at a concentration of 20 mM. Decreased cell viability was associated with increased intracellular calcium concentration and enhanced

apoptosis, as well as necrosis. Consistent with the findings of the present study, a recent study demonstrated that treatment with 20 mM glutamate induced apoptosis and elevated reactive oxygen species (ROS) generation in SH-SY5Y cells (13). RIP kinase 1 and RIP kinase 3 are key signaling molecules in programmed necrosis (9,10). The current study demonstrates that glutamate concentrations >20 mM significantly upregulate the expression of RIP kinase 1, but not RIP kinase 3, indicating that RIP kinase 1-mediated programmed necrosis may contribute to neuronal cell death in glutamate-mediated excitatory toxicity. To the best of our knowledge, there are only a small number of reports that indicate the involvement of RIP kinase 1 or RIP kinase 3 in neuronal damage; thus, future studies are required to investigate the precise mechanism of RIP-regulated neuronal death.

Neuroinflammatory processes are known to be involved in the pathogenesis of PD (14). The pro-inflammatory cytokine, 6-OHDA, a hydroxylated analogue of dopamine that activates an increase in ROS, is widely administered to establish cell and animal models of PD (15,16). In the current study, 6-OHDA suppressed cell viability, induced cell necrosis, elevated intracellular calcium concentrations and increased the mitochondrial membrane potential in SH-SY5Y cells. Notably, 6-OHDA treatment markedly elevated the intracellular calcium concentration compared with glutamate or glucose oxidase treatment, indicating that calcium influx and the subsequent intracellular calcium overload may contribute to 6-OHDA-induced neuronal cell death. Unlike glutamate treatment, the addition of 6-OHDA did not markedly alter the protein expression of RIP kinase 1 and RIP kinase 3 (data not shown), indicating RIPs may not be involved in 6-OHDA-mediated neuronal cell death. Elevated intracellular calcium concentration and mitochondrial membrane potential in SH-SY5Y cells may contribute to necrotic cell death. A previous study demonstrated that 6-OHDA treatment induced mitochondrial dysfunction in SH-SY5Y cells by activating the mitochondrial translocation of c-Jun N-terminal kinase (JNK) (17). Hence, other molecular signals, such as those of JNK, may be involved in 6-OHDA-mediated cytotoxicity in SH-SY5Y cells.

Oxidative stress is a contributing factor in neurological and neurodegenerative diseases, and is associated with aging (18). Glucose oxidase produces ROS and contributes to mitochondrial DNA damage through the generation of hydrogen peroxide. Glucose oxidase is, therefore, widely used to induce cellular toxicity by ROS *in vitro* (19). However, to the best of our knowledge, the effects of glucose oxidase on SH-SY5Y cells remain to be elucidated. In the present study, glucose oxidase treatment suppressed cell viability, induced early apoptosis, elevated intracellular calcium concentration and increased the mitochondrial membrane potential in SH-SY5Y cells. Oxidative stress did not alter expression of RIP kinase 1 and RIP kinase 3, indicating that these proteins may not be involved in oxidative stress-induced neuronal cell death. It is hypothesized that glucose oxidase leads to early apoptosis, and that increased intracellular calcium levels may induce irreversible rupture and cell death.

In conclusion, the present study demonstrates the different responses of SH-SY5Y cells to various stimuli. Thus, these SH-SY5Y neuronal cells may be utilized for the determination of therapeutic agent protection in different experimental paradigms. In addition, RIP kinase 1 may regulate programmed necrosis in glutamate-mediated excitatory toxicity, although not in cell damage induced by either 6-OHDA or glucose oxidase. This indicates that RIP kinase 1 may serve as a therapeutic target in the treatment of excitatory neurotransmitter-induced neurotoxicity in disorders of the CNS.

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