Free Radical Research, December 2005; 39(12): 1295–1304

Rac1-NADPH oxidase-regulated generation of reactive oxygen species mediates glutamate-induced apoptosis in SH-SY5Y human neuroblastoma cells

SEVDALINA NIKOLOVA[1], YONG SOO LEE[2], YOON-SEOK LEE[3], & JUNG-AE KIM[1,3]

[1]*Department of Biotechnology, Graduate School, Yeungnam University, Gyeongsan 712-749, South Korea,* [2]*College of Pharmacy, Duksung Women's University, Seoul 132-714, South Korea, and* [3]*College of Pharmacy, Yeungnam University, Gyeongsan 712-749, South Korea*

Accepted by Professor B. Halliwell

(Received 20 January 2005; in revised form 30 April 2005)

Abstract

Reactive oxygen species (ROS) are known to play an important role in glutamate-induced neuronal cell death. In the present study, we examined whether NADPH oxidase serves as a source of ROS production and plays a role in glutamate-induced cell death in SH-SY5Y human neuroblastoma cells. Stimulation of the cells with glutamate (100 mM) induced apoptotic cell death and increase in the level of ROS, and these effects of glutamate were significantly suppressed by the inhibitors of the NADPH oxidase, diphenylene iodonium, apocynin, and neopterine. In addition, RT-PCR revealed that SH-SY5Y cells expressed mRNA of gp91^{phox}, p22^{phox} and cytosolic p47^{phox}, p67^{phox} and p40^{phox}, the components of the plasma membrane NADPH oxidase. Treatment with glutamate also resulted in activation and translocation of Rac1 to the plasma membrane. Moreover, the expression of Rac1N17, a dominant negative mutant of Rac1, significantly blocked the glutamate-induced ROS generation and cell death. Collectively, these results suggest that the plasma membrane-bound NADPH oxidase complex may play an essential role in the glutamate-induced apoptotic cell death through increased production of ROS.

Keywords: *NADPH oxidase, Rac1, glutamate, apoptosis, reactive oxygen species, SH-SY5Y cells*

Abbreviations: *DCFH-DA, 2′,7′-dichlorofluorescin diacetate; DMSO, dimethyl sulfoxide; DPI, diphenylene iodonium; NAC, N-acetyl-L-cysteine; NDGA, nordihydroguaiaretic acid; NMDA, N-methyl-D-aspartate; PI, propidium iodide; ROS, reactive oxygen species; RT-PCR, reverse transcription polymerase chain reaction*

Introduction

Reactive oxygen species (ROS) have been recognized as important signaling molecules inducing apoptosis [1–3], mitogenesis, inflammation [4] and differentiation [5] depending on the concentration. In particular, ROS and consequent oxidative stress have been shown to play an essential role in glutamate toxicity which contributes to the neuronal loss associated with both acute insults like ischemia [6,7] and chronic neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS), Alzheimer's disease, Parkinson's disease, Huntington's disease and neuronal acquired immunodeficiency syndrome [8].

The biological processes leading to ROS generation are electron transports associated with mitochondrial membranes as well as non-mitochondrial enzymes such as cytochrome P-450, cyclooxygenase, nitric oxide synthase, xanthine oxidase and ribonucleotide reductase [9,10]. In addition, the membrane-bound NADPH oxidase is known to produce ROS during the respiratory burst in neutrophils. The activation of this enzyme proceeds through a multistep assembly at the plasma membrane of several components including the two subunits of cytochrome b_{558}

Correspondence: J. A. Kim, College of Pharmacy, Yeungnam University, Gyeongsan 712-749, South Korea. Tel: 82 53 810 2816. Fax: 82 53 810 4654. E-mail: jakim@yu.ac.kr

ISSN 1071-5762 print/ISSN 1029-2470 online © 2005 Taylor & Francis
DOI: 10.1080/10715760500176866

($p22^{phox}$ and $gp91^{phox}$), the small GTP-binding proteins (Rac and Rap1A), and the cytosolic factors ($p40^{phox}$, $p47^{phox}$ and $p67^{phox}$) [11,12]. Although the NADPH oxidase was first characterized in white blood cells such as neutrophils, it has recently been found to be far more widespread being present in cells that have no role in host defense. For example, the NADPH oxidase components exist in fibroblasts [13], mesangial cells [14], endothelial cells [15], osteoclasts [16] and chondrocytes [17,18]. A new family of homologues of the 91 kDa glycoprotein ($gp91^{phox}$) containing seven members and functioning in proton transport has recently been identified in various cell types. The NADPH oxidase (NOX)/dual oxidase (DUOX) family has been detected in the epithelium, smooth-muscle cells and the endothelium [19]. Homologues of $gp91^{phox}$ have also been cloned from plants [20,21]. Such wide distribution of the NADPH oxidase complex implies a role other than simply host defense. Whereas ROS generation during phagocytosis appears to occur in the extracellular (phagosomal) compartments, ROS generation through nonphagocytic NADPH oxidase seems to happen intracellularly and upregulation of this intracellular ROS source may contribute to oxidative stress [22].

Glutamate excitotoxicity is, in large part, mediated by *N*-methyl-D-aspartate (NMDA) receptor activation and subsequent increase in intracellular calcium, nitric oxide and free radicals. Activation of phospholipase A2 through glutamate-stimulated NMDA receptors releases arachidonic acid (AA). The metabolic process of AA by cyclooxygenase and lipoxygenase leads to generation of ROS [23]. In addition, AA is known to promote the assembly of phagocytic NADPH oxidase subunits triggering the activation of the complex [24]. However, the exact role of the nonphgocytic NADPH oxidase in the glutamate-induced toxicity in human neuronal cells have not been reported yet. Hence, in the present study, we investigated whether the NADPH oxidase system is present and responsible for glutamate-induced apoptotic neuronal cell death using SH-SY5Y human neuroblastoma cells which have been shown to express both ionotrophic and metabotrophic glutamate receptors [25–27].

Materials and methods

Materials

Powered Dulbecco's Modified Eagle Medium: Nutrient Mixture F12 (DMEM-F12), trypsin solution, L-glutamic acid (glutamate), DPI, apocynin, neopterine, PI, ribonuclease A, protease inhibitor cocktail and all salt powders were obtained from Sigma Chemical CO. (St. Louis, MO, USA). DCFH-DA was from Molecular Probes, Inc. (Eugene, OR). The plasmid vectors pEXV, pEXVmyc-rac1N17 and pEXVmyc-rac1WT were generously provided by Dr Yun Soo Bae (Ewha Women's University, Seoul, South Korea). pCMV-β-gal vector was from BD Bioscience (Clontech). FuGene6 transfection solution was from Roche (Mannheim, Germany). All used PCR primers were ordered from Bioneer (Daejeon, Korea) and Taq DNA polymerase was from Promega (Madison, USA). Fetal bovine serum (FBS) and antibiotics (penicillin and streptomycin mixture) were purchased from GIBCO (Grand Island, NY). Glutamate was dissolved in serum free DMEM-F12 media prior to use. DCFH-DA was prepared as stock solutions in dimethyl sulfoxide (DMSO), then diluted with aqueous medium to the final desired concentrations. The stock solutions of drugs were sterilized by filtration through 0.2 μm disc filters (Gelman Sciences, Ann Arbor, MI).

Cell line and cell culture

SH-SY5Y human neuroblastoma cell line was purchased from American Type Culture Collection (Manassas, VA). SH-SY5Y cells were cultured in DMEM F-12 medium supplemented with 10% FBS, 2.44 g/l sodium bicarbonate, 15 mM HEPES, 100 units penicillin/ml and 100 μg streptomycin/ml at 37°C in a humid atmosphere with 5% CO_2. Cells were split in a 1:2 ratio every 5 days by trypsinization.

Flow cytometric analysis of apoptosis

To determine apoptosis, SH-SY5Y cells were stained with PI. At the end of glutamate incubation, SH-SY5Y cells were trypsinized, washed twice with cold PBS and then fixed with 70% ethanol for 30 min at 4°C. The fixed cells were washed twice with PBS, incubated in PBS containing 100 μg/ml ribonuclease A for 30 min at 37°C, stained with 50 μg/ml PI for 30 min and then filtered through a 40 μm nylon mesh filter (Falcon, USA). The cells were subjected to flow cytometric analysis using a FACSCalibur (Becton Dickinson, USA) equipped with CellQuest program.

Intracellular ROS measurement

Relative changes in intracellular ROS in the SH-SY5Y cells were monitored using a fluorescent probe DCFH-DA. Aliquots of the SH-SY5Y cells were washed in EBSS. Then, 5 μM Fura-2 was added, and the cells were incubated for 30 min at 37°C. Unloaded Fura-2 was removed by centrifugation at 150*g* for 3 min. Cells were resuspended at a density of 2×10^6 cells/ml in Krebs-Ringer buffer containing 125 mM NaCl, 5 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM KH_2PO_4,

1.2 mM $MgSO_4$, 5 mM $NaHCO_3$, 25 mM HEPES, 6 mM glucose and 2.5 mM probenecid (pH 7.4). Fura-2-loaded cells were maintained at 25°C for 90 min before fluorescence measurement. For each experiment, 0.5 ml aliquot of Fura-2-loaded cells was equilibrated to 37°C in a stirred quartz cuvette. Fluorescence emission (510 nm) was monitored with the excitation wavelength cycling between 340 and 380 nm using a Hitachi F4500 fluorescence spectrophotometer. At the end of an experiment, fluorescence maximum and minimum values at each excitation wavelength were obtained by lysis of cells with 20 μg/ml digitonin (maximum) and then adding 10 mM EGTA (minimum). With the maximum and minimum values, the 340:380 nm fluorescence ratios were converted into free Ca^{2+} concentrations using a software, F-4500 Intracellular Cation Measurement System, provided by Hitachi.

RT-PCR

Total cellular RNA was extracted from SH-SY5Y cells with RNeasy kit (Qiagene, Hilden, Germany). The production of cDNA was carried out using Ready-to-go T-primed first strand kit (Amersharm Biosciences, Buckinghamshire, England), by incubating 5 μg of RNA, following the manufactured instructions. The polymerase chain reaction was performed with 0.1 μg cDNA using 0.5 μM primers listed in Table I in the presence of 0.5 U Taq DNA polymerase (Promega, Madison, USA). Table I includes also the annealing temperature and the number of cycles for each amplification reaction. The PCR products were visualized by electrophoresis on a 2% agarose gel stained with ethidium bromide.

Rac activation assay

SH-SY5Y cells, starved for 24 h by exposing to DMEM/F12 media containing 1% serum, with or without glutamate were lysed. Total cellular protein (150 μg) was incubated with 10 μg of agarose beads containing p21-binding domain (PBD) of p21-activated protein kinase 1 (PAK1), an effector of activated Rac, for 1 h at 4°C. The beads were collected by centrifugation and washed two times in the lysis buffer. The beads were resuspended in sample buffer and boiled for 5 min. Proteins were resolved by SDS-PAGE using a 15% gel, transferred electrophoretically and visualized using anti-human Rac antibody and electrochemoluminescence (ECL) kit. For the positive control the nonhydrolyzable GTP analog GTPγS was used according to the manufacturer's protocol (Upstate, New York).

Subcellular fractionation and Western blot analysis

The cells were washed twice with ice-cold PBS and homogenized in a lysis buffer (10 mM HEPES, 10 mM NaCl, 1 mM KH_2PO_4, 5 mM $NaHCO_3$, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5 mM EDTA, 1 mM PMSF and protease inhibitor cocktail, Triton X-100). The lysates were centrifuged at 33,000 *g* for 5 min at 4°C, and supernatant proteins were ultracentrifuged at 110,000 *g* for 1 h at 4°C. The supernatant containing cytosolic proteins and pellet containing membrane protein were separated on 15% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane at 200 mA for 1 h. After incubation with a polyclonal antibody against human Rac1 (Santa Cruz Biotechnology, Santa Cruz, CA), signals were visualized using anti-rabbit IgG and an ECL nitrocellulose membrane (Amersham Biosciences).

Transient transfection assay

Cells at 60% confluence were transiently transfected with pEXV-myc-N17rac1, pEXVmyc-wtrac1 or empty pEXV vector with FuGENE6 transfection

Table I. The sequences of sense and anti-sense primers used in reverse transcription polymerase chain reaction (RT-PCR).

Genes (bp product)	Annealing temperature (°C)	Primer sequence	No. of cycles
$P67^{phox}$ (574)	60	5′-GGAGTGTGTCTGGAAGCAG-3′ 3′-AGTGTGGTAGGGCATGGGAAC	45
$P47^{phox}$ (241)	60	5′-GATCAATCCAGAGAACAGGA-3′ 3′-ATCTTTGGGCATCAAGTATG-5′	40
$p40^{phox}$ (380)	57	5′-GTCTGGGTGCTGATGGATGA-3′ 3′-TCTTCGTAGTAGTAGCAACG-5′	45
Rac1 (348)	62	5′-GGATACAGCTGGACAAGAAG-3′ 3′-TTTTACAGCACCAATCTCCT-5′	45
$p22\text{-}^{phox}$ (317)	64	5′-GTTTGTGTGCCTGCTGGAGT-3′ 3′-TGGGCGGCTGCTTGATGGT-5′	45
$gp91\text{-}^{phox}$ (404)	63.5	5′-GCTGTTCAATGCTTGTGGCT-3′ 3′-TCTCCTCATCATGGTGCACA-5′	45
GAPDH (450)	58	5′-GGTGAAGGTCGGAGTCAACG-3′ 3′-CAAAGTTGTCATGGATGACC-5′	40

solution (Roche) in a serum free medium for 24 h. After the medium was changed to fresh full growth medium, cells were further incubated for 24 h, then, treated with glutamate. The transfection efficiency was 21.40 ± 0.98%, normalized by cotransfection with pCMVβ vector (Clontech).

In order to confirm the transient transfection efficiency, SH-SY5Y cells were cotransfected with pCMVβ. β-galactosidase activity in transiently transfected SH-SY5Y cells was assayed with β-galactosidase staining kit (Intron Biotechnology, Korea). Transfected SH-SY5Y cells were fixed for 10 min at room temperature with glutaraldehyde–formaldehyde and exposed to a X-Gal staining solution for 3 h. The transfection efficiency was determined by counting stained cells under a microscope and expressed as the percentage of stained cells in the total population.

Data analysis

Data were expressed as means ± standard error of the mean (SEM) and were 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

Role of NADPH oxidase in the glutamate-induced ROS generation and apoptosis in SH-SY5Y human neuroblastoma cells

Glutamate stimulation of SH-SY5Y human neuroblastoma cells induced apoptosis in a concentration- and time-dependent manner studied by flow cytometry by determining a loss of phospholipid asymmetry detected by annexin-V binding and the hypodiploid DNA content stained with PI as depicted in Figure 1A and B, respectively. Since glutamate toxicity is largely mediated through intracellular Ca^{2+} increase and the generation of ROS, we examined the effect of a ROS scavenger on glutamate-induced cell death. As shown in Figure 2A and B, the level of ROS in glutamate-treated cells was increased. Furthermore, *N*-acetylcysteine (NAC), an antioxidant, significantly blocked the glutamate-induced cell death as depicted in Figure 2C.

In order to clarify the site of ROS production and its role in the glutamate-induced cell death, we investigated the effects of various drugs that inhibit the mitochondrial respiratory chain enzymes or non-mitochondrial ROS-generating enzymes, on the glutamate-induced ROS generation and apoptosis. The effects of glutamate (100 mM) on the ROS level

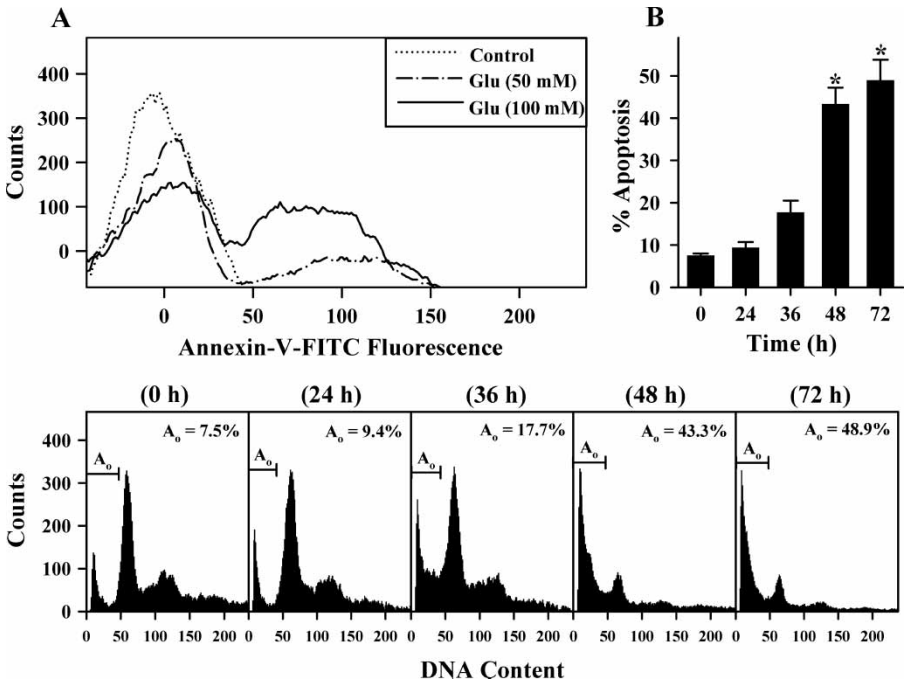

Figure 1. Glutamate-induced apoptosis of SH-SY5Y cells. In the experiments of (A) the cells were incubated in the absence (control) or in the presence of glutamate for 48 h. Cells were stained with annexin-V-FLUOS and analyzed by flow cytometry. Note that in the presence of glutamate there is a shift in annexin-V-FLUOUS fluorescence. This is due to the binding of annexin-V to membrane phospholipids of cells undergoing apoptosis. Results are representative of four experiments. In the experiments of (B), the cells were incubated with glutamate (100 mM) for each designated time. The number of apoptotic cells was measured by flow cytometry as described in text. The region to the left of the G_o/G_1 peak was defined as cells undergoing apoptosis-associated DNA degradation. In bar graphs, the data represent the mean values of four replications with bars indicating SEM. $^*P < 0.05$ compared to control.

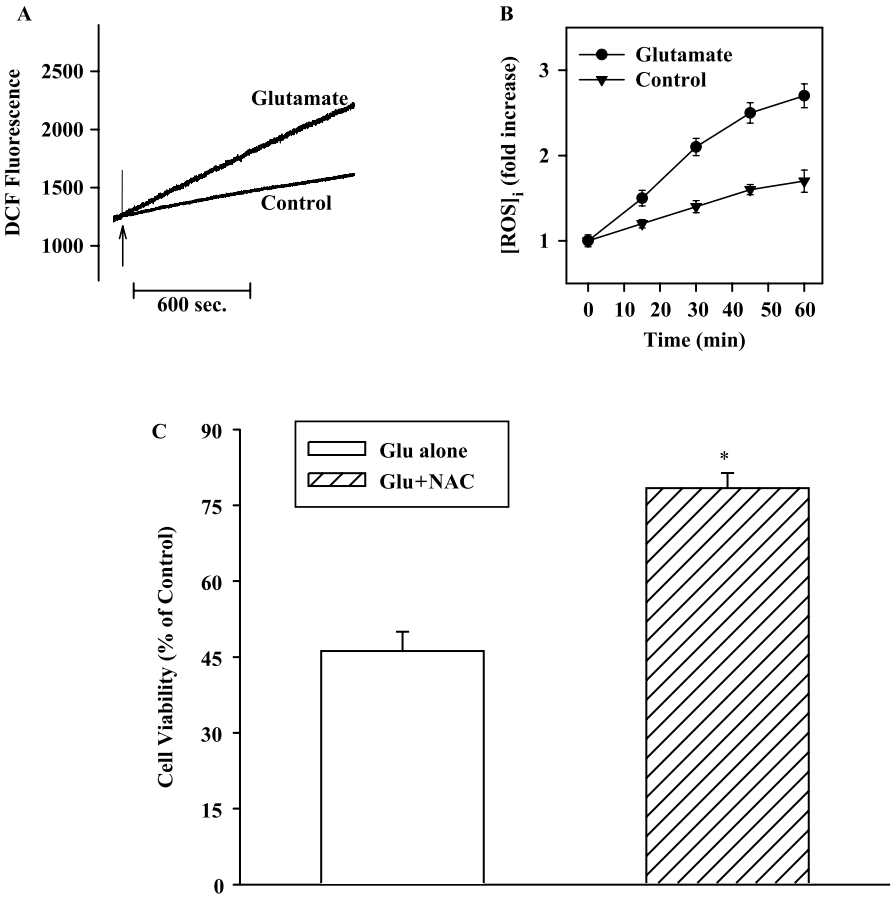

Figure 2. Glutamate increases ROS generation and NAC, an antioxidant, prevents glutamate-induced cell death. The data (A) show changes in ROS levels as a function of time, which was measured by DCF fluorescence method. The arrow shows the time point for addition of glutamate (100 mM). In the data (B) results are expressed as fold increase compared to the initial DCF fluorescence intensity. In the experiments of (C) cells treated for 48 h with or without glutamate were analyzed for viability by MTT assay. NAC (2 mM) were added 30 min prior to glutamate treatment. Data points represent the mean values of four replications with bars indicating SEM. $^{*}P < 0.05$ compared to glutamate alone.

and apoptosis in SH-SY5Y cells were not significantly altered neither by rotenone (10 μM), an inhibitor of the mitochondrial electron transport chain, nor by allopurinol (100 μM), an inhibitor of xanthine oxidase, implying that these enzymes may not be actively involved in the glutamate effects (Table II). In contrast, the glutamate-induced ROS generation was completely suppressed by inhibitors of the membrane-bound NADPH oxidase, 5 μM DPI, 10 μM apocynin and 100 μM neopterine as shown in Figure 3A and B. Furthermore, the glutamate-induced apoptotic cell death tested by flow cytometry by determining hypodiploid DNA content of PI stained nuclei was also significantly blocked by these inhibitors of the NADPH oxidase, as depicted in Figure 3C and D. ROS production by glutamate may also be not mediated through the NMDA receptor-stimulated arachdonic acid (AA) release, since indomethacin and NDGA, inhibitors of AA metabolism, did not significantly block the glutamate-induced ROS increase and apoptosis, as shown in Table II. These results suggest that NADPH oxidase complex may act as a major source of the glutamate-induced ROS generation, and it may play a major role in the glutamate-induced apoptosis in SH-SY5Y cells.

Presence of the NADPH oxidase complex in SH-SY5Y human neuroblastoma cells. The presence and activity of the NADPH oxidase complex similar to that found in neutrophils has been reported in many cell types including rodent neurons, in which NADPH oxidase contributes to nerve growth factor-deprived cell death [28] and ischemic brain injury [29]. However, neuronal cells derived from human brain have not been found so far to express this multicomponent enzyme complex. Thus we verified the presence of each component of the enzyme in the cells using RT-PCR method. Activation of the NADPH oxidase requires assembly of five subunit components, the two

Table II. Effects of various inhibitors of ROS-generating enzymes on the glutamate-induced ROS generation and apoptosis in SH-SY5Y cells.

	$[ROS]_i$ (% increase)†		% Apoptosis‡	
Compound	− glutamate	+ glutamate	− glutamate	+ glutamate
None	119.4 ± 8.2	210.6 ± 53.5*	4.2 ± 0.6	40.2 ± 3.2*
Rotenone (10 μM)	114.1 ± 15.7	232.4 ± 32.1*	8.2 ± 1.5	44.4 ± 5.8*
Allopurinol (100 μM)	139.9 ± 13.2	191.1 ± 32.3*	8.6 ± 1.3	48.2 ± 5.3*
Indomethacin (100 μM)	137.2 ± 12.1	216.1 ± 27.1*	6.4 ± 2.4	51.7 ± 6.3*
NDGA (10 μM)	123.4 ± 11.5	201.8 ± 22.7*	15.6 ± 2.3	53.2 ± 7.3*

All data represent the mean values ± SEM. $^*P < 0.05$ compared to control.
† $[ROS]_i$ was measured by DCF fluorescence method, and the values are expressed as percent increase of DCF fluorescence intensity induced by treatment with or without glutamate (100 mM) for 1 h compared to that of initial time. Each drug was added 10 min before glutamate treatment. ‡Apoptosis was measured by flow cytometry. In the experiments the cells were incubated with glutamate (100 mM) for 48 h. Each drug was added 30 min before glutamate treatment.

subunits of cytochrome *b*558 ($p22^{phox}$ and $gp91^{phox}$) and the cytosolic factors ($p40^{phox}$, $p47^{phox}$ and $p67^{phox}$) at the plasma membrane [12]. As illustrated in Figure 4, we detected all five mRNAs encoding the NADPH oxidase components in SH-SY5Y human neuroblastoma cells. This and the above results strongly suggest that the NADPH oxidase complex may be actively involved in the glutamate-induced ROS generation in the cells.

Glutamate activates and translocates cytosolic Rac to the plasma membrane in SH-SY5Y cells

The above data further prompted us to investigate whether stimulation of SH-SY5Y cells with glutamate results in direct activation of Rac. During recent years, several mechanisms have been proposed for the activation of small G proteins, including guanine nucleotide exchange factors which facilitate

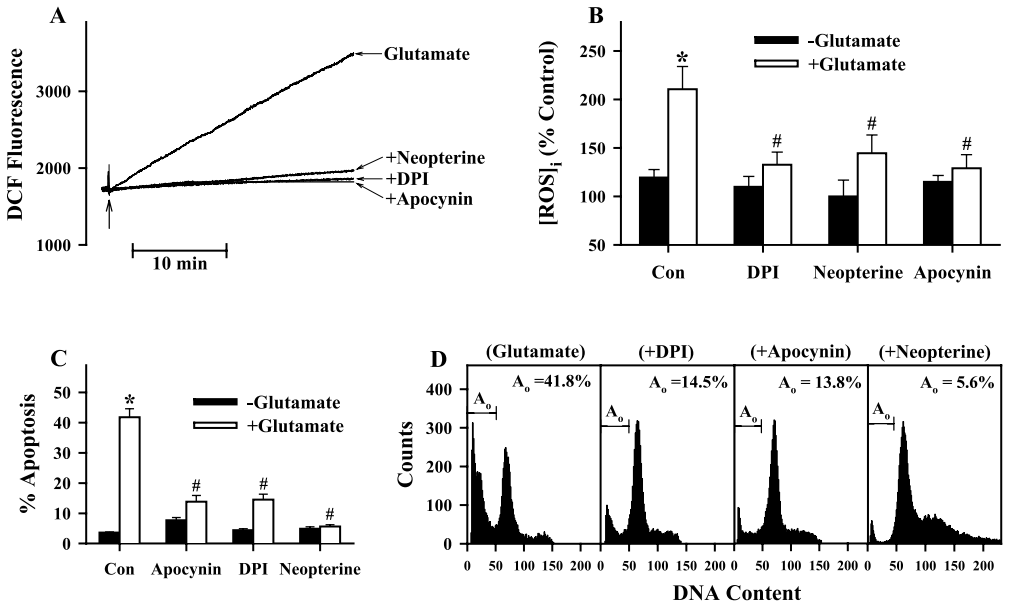

Figure 3. Effects of NADPH oxidase inhibitors on the ROS generation (A and B) and apoptosis (C and D) induced by glutamate in SH-SY5Y human neuroblastoma cells. In these experiments DPI (5 μM), apocynin (10 μM) and neopterine (100 μM) were used. These drugs were given 10 min before glutamate (100 mM) application. The data (A) show changes in ROS levels as a function of time, which was measured by DCF fluorescence method. The arrow shows the time point for addition of glutamate (100 mM). In the data (B) results are expressed as fold increase compared to the initial DCF fluorescence intensity. In the experiments of (C and D) the cells were incubated with glutamate (100 mM) for 48 h. The inhibitors were added 30 min before glutamate treatment. The number of apoptotic cells was measured by flow cytometry. In bar graphs the data represent the mean values of four replications with bars indicating SEM. $^*P < 0.05$ compared to control. $^{\#}P < 0.05$ compared to glutamate alone.

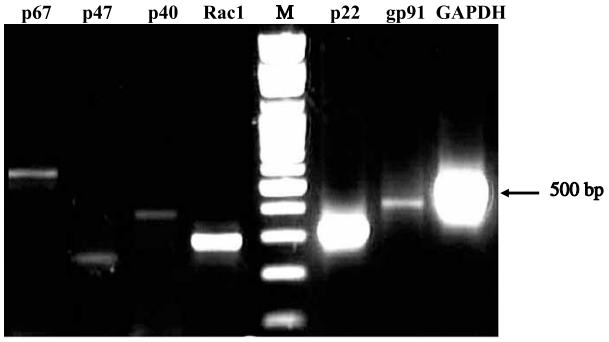

Figure 4. mRNA expression of NADPH oxidase subunits in SH-SY5Y human neuroblastoma cells. The expression of each NADPH oxidase subunit at the mRNA level was detected by performing a fixed number of PCR cycles, listed in Table I, on 0.1 μg cDNA generated from total RNA isolated from the cells. The PCR products were visualized by electrophoresis on a 2% agarose gel stained with ethidium bromide. M: 100 bp DNA ladder size marker.

replacement of a GTP for a GDP molecule on the G proteins [30]. Such factor (e.g. Tiam 1) has been identified for Rac. However, determination of Rac activation via Tiam 1 is difficult, because numerous other exchange factors to activate Rac singly or through the intermediacy of other G proteins have also been identified. Therefore, in the present study, we estimated the degree of glutamate-induced activation of Rac (i.e. its GTP-bound form) in a pull-down assay using a PAK, an effecter of activated Rac, binding method. The data shown in Figure 5A demonstrate a marked increase in the binding degree of activated Rac to PAK in lysates of SH-SY5Y following exposure to glutamate. As a positive control, we used GTP-γS, a non-hydrolyzable analog of GTP, to facilitate binding and activation of endogenous Rac in the SH-SY5Y cells. We observed higher binding of Rac to PAK under these conditions compared with the glutamate-treated cell lysates. Our results also revealed stable level of the expression of total Rac upon glutamate treatment. Together, these data suggest that glutamate-induced SH-SY5Y cell death may involve activation of Rac, presumably via stimulation of GTP/GDP exchange [31].

Activated Rac (GTP-bound form) subsequently translocates to the plasma membrane [32], leading to the activation of the NADPH oxidase complex. In the next series of experiments, we examined whether exposure of SH-SY5Y cells to glutamate induces the membrane translocation of Rac. Glutamate translocated cytosolic Rac1 to the plasma membrane, which was revealed by immunocytochemistry and confirmed by disappearance of Rac1 protein in the

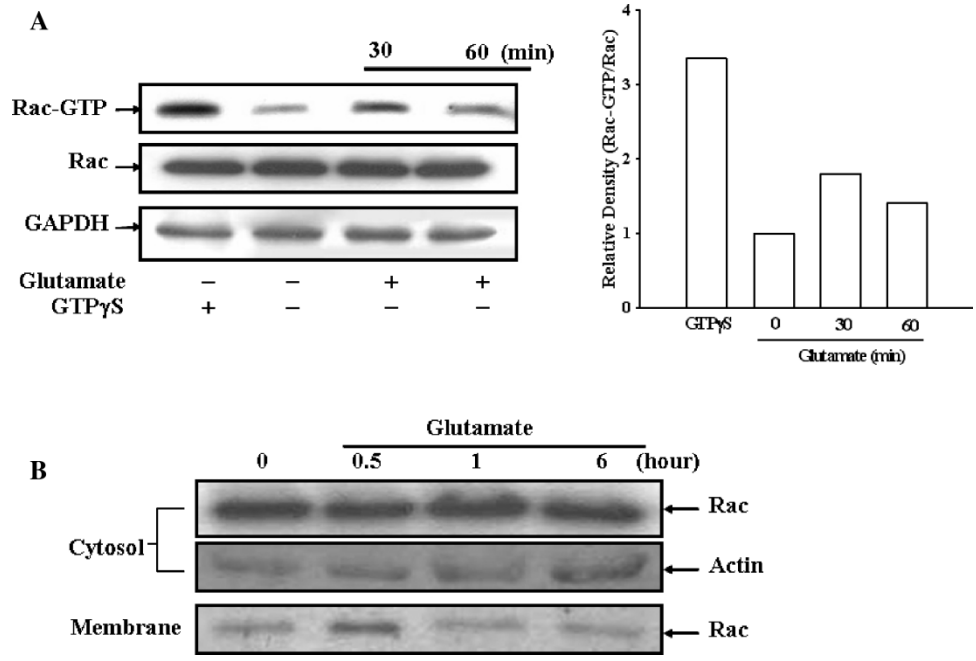

Figure 5. Glutamate-induced activation and translocation of Rac in the cells. In (A) activated GTP-bound Rac was purified using affinity binding beads which contain p21-binding domain (PBD) of p21-activated protein kinase 1 (PAK1), an effector of Rac. In the upper blot, the lysates of SH-SY5Y cells were either loaded with 100 μM GTPγS (at 30°C for 15 min) or stimulated with 100 mM glutamate for the times indicated. As evident from the blot *in vitro* GTPγS protein loading activated much higher percentage of the Rac. The middle blot shows the total amount of Rac (independent of the activation state) present in an aliquot of the cell lysates before bead addition. The bottom blot represents GAPDH used as a control for equal loading. The bar graph represent the relative density of the signal. In (B) fractionated cytosolic and membrane proteins were immunoblotted with Rac1 antibody.

cytoplasm and increased Rac1 protein level in the membrane assessed by Western blot analysis (Figure 5B). This membrane binding of Rac correlates with the mechanisms of the activation of the NADPH oxidase [26], which reinforces the possibility that Rac-NADPH oxidase signaling pathway may play an important role in the glutamate-induced generation of ROS and apoptosis in SH-SY5Y cells.

Transient expression of dominant negative Rac markedly reduces glutamate-induced cell death

To further confirm that Rac is a key regulatory protein in glutamate-induced SH-SY5Y cell death, we studied the effects of glutamate on cell death in the SH-SY5Y cells transiently expressing a dominant negative mutant of Rac1 (N17Rac1), wild-type Rac1 (wtRac1) and the vector alone. As shown in Figure 6, we found a significant reduction in glutamate-induced apoptotic cell death in the SH-SY5Y cells expressing the dominant negative form of Rac1, which is compatible with the glutamate-induced Rac-GTP formation and Rac membrane translocation.

Discussion

Glutamate toxicity has been implicated in many aspects of brain injury including traumatic, ischemic and hemorrhagic damage. A salient feature in glutamate-induced toxicity is the induction of oxidative burst and the subsequent oxidative stress. Whereas multiple enzymes and processes can contribute to oxidative stress, recent studies indicate that a multicomponent phagocyte-type NADPH oxidase is a major source of ROS production in many nonphagocytic cells, including fibroblasts, vascular smooth muscle cells, endothelial cells, renal mesangial cells and tubular cells [22]. Thus in the present study we targeted one new and potential source of ROS, the NADPH oxidase complex, in the face of glutamate-induced neurodegeneration. In the present study, we detected the presence of NADPH oxidase complex in human brain-derived neuronal cells. SH-SY5Y cells used in the current study as a cellular model were found to express all the components ($p22^{phox}$, $gp91^{phox}$, $p40^{phox}$, $p47^{phox}$, $p67^{phox}$ and Rac1/2) of NADPH oxidase complex (Figure 4). The significant suppression of the glutamate-induced production of ROS (Figure 3A and B) by various inhibitors of the

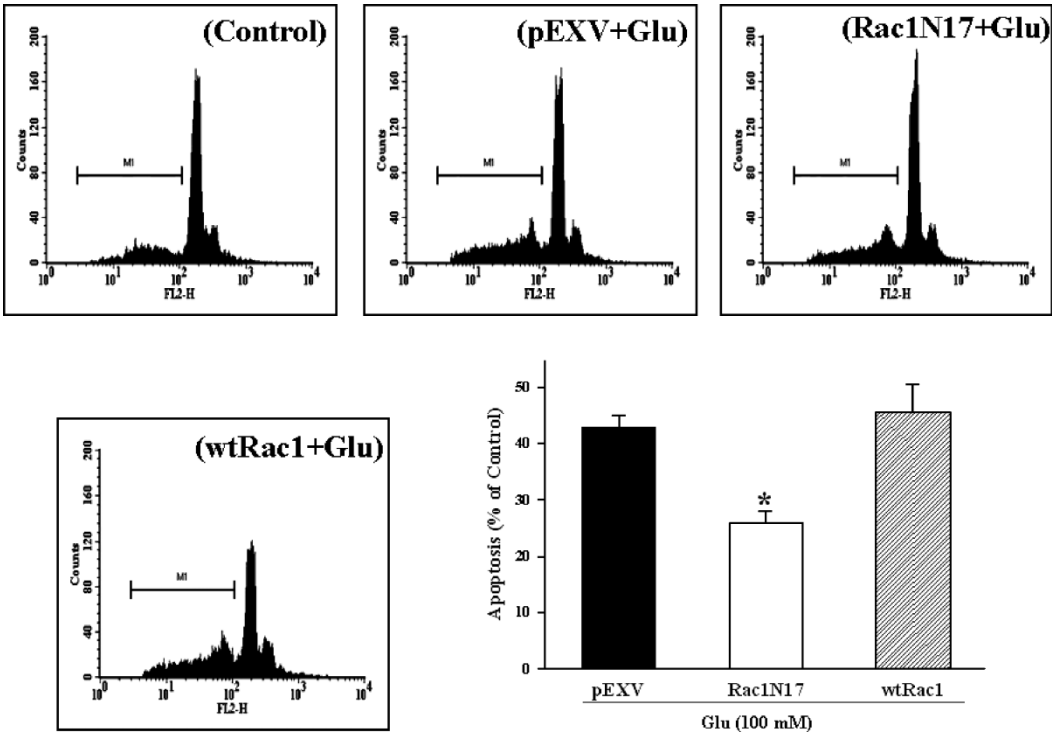

Figure 6. Transfection with a dominant-negative mutant of Rac1 (N17Rac1) reverses the apoptotic effect of glutamate in SH-SY5Y cells. Cells were transfected with pEXVmycrac1N17, pEXVmyc-rac1WT and empty pEXV vector. Apoptosis was measured at 48 h after glutamate treatment by flow cytometry the same manner as described in Figure 1. In these experiments 100 mM of glutamate was used. $*P < 0.05$ compared to vector alone.

enzyme (DPI, apocynin, neopterin) suggests that the membrane-bound NADPH oxidase complex may be actively involved in the production of ROS by glutamate. Similar results have been found in β-amyloid-treated glial cells [33]. Based on the known mechanisms [28], glutamate-induced activation of the NADPH oxidase may primarily involve Rac-GDP/GTP exchange. Data from our pull-down assay for PAK-Rac binding are more supportive for the glutamate-induced activation (i.e. formation of the GTP bound form) of Rac in SH-SY5Y cells (Figure 5A). In confirmation of this, we also showed that glutamate treatment of SH-SY5Y cells resulted in the translocation of Rac1 to the plasma membrane (Figure 5B). Furthermore, we observed a reduction in the glutamate-induced apoptotic cell death in SH-SY5Y cells transiently expressing N17Rac1, the dominant negative mutant of Rac1 (Figure 6). Therefore, it is very likely that glutamate-induced SH-SY5Y cell death may involve Rac-NADPH oxidase activation.

It is known that AA acts as a mediator for promoting the assembly of phagocytic NADPH oxidase subunits, triggering out the activation of the complex. AA has been reported to translocate Rac from cytosol to the plasma membrane [34] and phosphorylate $p47^{phox}$ by protein kinase C signaling pathway [35]. We also tested the effects of inhibitors of AA metabolism (indomethacin and NDGA) on the glutamate-induced ROS production. We found that these inhibitors had no influence (Table II), suggesting that AA metabolism may not be involved in the NADPH oxidase-mediated ROS formation induced by glutamate.

In this study, we did not determine the upstream mechanism by which glutamate activates the NADPH oxidase. Since it is known that glutamate toxicity is mediated through NMDA receptors and that there is an interaction of NMDA receptor with dopamine receptor and/or metabotropic glutamate receptors [25,26], further study is required to unravel the signaling pathway to the activation of NADPH oxidase in relation to the glutamate receptors.

In summary, these data provide an evidence that the glutamate-induced neuronal cell death involves ROS produced by activation of the plasma membrane-bound NADPH oxidase complex in SH-SY5Y human neuroblastoma cells, and that the a small G-protein, Rac, is a key regulator in this process. Therefore, modulation of this enzymatic complex could be a promising step to prevent oxidative stress implicated in glutamate-toxicity. Possible therapeutic approaches may include the use of antioxidants and direct pharmaceutical or genetic manipulation of the NADPH oxidase.

Acknowledgements

This work was supported by grant from the Basic Research Program of the Korea Science and Engineering Foundation (No. R04-2000-000-00058-0).

References

[1] Gardner AM, Xu FH, Fady C, Jacoby FJ, Duffey DC, Tu Y, Lichtenstein A. Apoptotic vs. nonapoptotic cytotoxicity induced by hydrogen peroxide. Free Radic Biol Med 1997; 22:73–83.

[2] Jayanthi S, Ordonez S, McCoy MT, Cadet JL. Dual mechanism of Fas-induced cell death in neuroglioma cells: A role for reactive oxygen species. Mol Brain Res 1999; 72:158–165.

[3] Hildeman DA, Mitchell T, Teague TK, Henson P, Day BJ, Kappler J, Marrack PC. Reactive oxygen species regulate activation-induced T cell apoptosis. Immunity 1999; 10:735–744.

[4] Irani K. Oxidant signaling in vascular cell growth, death, and survival: A review of the roles of reactive oxygen species in smooth muscle and endothelial cell mitogenic and apoptotic signaling. Circ Res 2000;87:179–183.

[5] Suzukawa K, Miura K, Mitsushita J, Resau J, Hirose K, Crystal R, Kamata T. Nerve growth factor-induced neuronal differentiation requires generation of Rac1-regulated reactive oxygen species. J Biol Chem 2000;275:13175–13178.

[6] Komara JS, Nayini NR, Bialick HA, Indrieri RJ, Evans AT, Garritano AM, Hoehner TJ, Jacobs WA, Huang RR, Krause GS, White BC, Aust SD. Brain iron delocalization and lipid peroxidation following cardiac arrest. Ann Emerg Med 1986; 15:384–389.

[7] Lazzarino G, Vagnozzi R, Tavazzi B, Pastore FS, Di Pierro D, Siragusa P, Belli A, Giuffre R, Giardina B. MDA, oxypurines, and nucleosides relate to reperfusion in short-term incomplete cerebral ischemia in the rat. Free Radic Biol Med 1992; 13:489–498.

[8] Greenamyre JT, Penney JB, Young AB, D'Amato CJ, Hicks SP, Shoulson I. Alterations in L-glutamate binding in Alzheimer's and Huntington's diseases. Science 1985; 227:1496–1499.

[9] Halliwell B. Free radicals, reactive oxygen species and human disease: A critical evaluation with special reference to atherosclerosis. Br J Exp Pathol 1989;70:737–757.

[10] Yu BP. Cellular defenses against damage from reactive oxygen species. Physiol Rev 1994;74:139–162.

[11] Babior BM. The respiratory burst oxidase. Curr Opin Hematol 1995;2:55–60.

[12] Babior BM. NADPH oxidase: An update. Blood 1999;93:1464–1476.

[13] Meier B, Cross AR, Hancock JT, Kaup FJ, Jones OTG. Identification of a superoxide-generating NADPH oxidase system in human fibroblasts. Biochem J 1991;275:241–245.

[14] Radeke HH, Cross AR, Hancock JT, Jones OTG, Nakamura M, Resch K. Functional expression of NADPH oxidase components (alpha- and beta-subunits of cytochrome b558 and 45-kDa flavoprotein) by intrinsic human glomerular mesangial cells. J Biol Chem 1991;266:21025–21029.

[15] Jones SA, O'Donnell VB, Wood JD, Broughton JP, Hughes EJ, Jones OTG. Expression of phagocyte NADPH oxidase components in human endothelial cells. Am J Physiol 1996;271:1626–1634.

[16] Steinbeck MJ, Appel WH, Verhoeven AJ, Karnovsky MJ. NADPH oxidase expression and *in situ* production of

superoxide by osteoclasts actively resorbing bone. J Cell Biol 1994;126:765–772.
[17] Hiran TS, Moulton PJ, Hancock JT. Detection of superoxide and NADPH oxidase in procine articular chondrocytes. Free Radic Biol Med 1997;23:736–743.
[18] Moulton PJ, Hiran TS, Goldring MB, Hancock JT. Detection of protein and mRNA of various components of the NADPH oxidase complex in an immortalized human chondrocyte line. Br J Rheumatol 1997;36:522–529.
[19] Lambeth JD. Nox enzymes and the biology of reactive oxygen. Nat Rev Immunol 2004;4:181–189.
[20] Desikan R, Burnett EC, Hancock JT, Neill SJ. Harpin and hydrogen peroxide induce the expression of a homologue of gp91-phox in Arabidopsis thaliana suspension cultures. J Exp Bot 1998;49:1767–1771.
[21] Torres MA, Onouchi H, Hamada S, Machida C, Hammond-Kosack KE, Jones JDG. Six Arabidopsis thaliana homologues of the human respiratory burst oxidase (gp91phox). Plant J 1998;14:365–370.
[22] Li J-M, Shah AM. ROS generation by nonphagocytic NADPH oxidase: Potential relevance in diabetic nephropathy. J Am Soc Nephrol 2003;14:221–226.
[23] Cui X-L, Douglas JG. Arachidonic acid activates c-jun N-terminal kinase through NADPH oxidase in rabbit proximal tubular epithelial cells. Proc Natl Acad Sci USA 1997;94:3771–3776.
[24] Shiose A, Sumimoto H. Arachidonic acid and phosphorylation synergistically induce a conformational change of p47phox to activate the phagocyte NADPH oxidase. J Biol Chem 2000;275:13793–13801.
[25] Nair VD, Niznik HB, Mishra RK. Interaction of NMDA and dopamine D2L receptors in human neuroblastoma SH-SY5Y cells. J Neurochem 1996;66:2390–2393.
[26] Naarala J, Nykvist P, Tuomala M, Savolainen K. Excitatory amino acid-induced slow biphasic responses of free intracellular calcium in human neuroblastoma cells. FEBS Lett 1993;330:222–226.
[27] Sun D, Murali SG. Stimulation of Na^+-K^+-$2Cl^-$ cotransporter in neuronal cells by excitatory neurotransmitter glutamate. Am J Physiol 1998;275:C772–C779.
[28] Tammariello SP, Quinn MT, Estus S. NADPH oxidase contributes directly to oxidative stress and apoptosis in nerve growth factor-deprived sympathetic neurons. J Neurosci RC53 2000;20:1–5.
[29] Vallet P, Charnay Y, Steger K, Ogier-Denis E, Kovari E, Herrmann F, Michel JP, Szanto I. Neuronal expression of the NADPH oxidase NOX4, and its regulation in mouse experimental brain ischemia. Neuroscience 2005; 132:233–238.
[30] Bokoch GM, Diebold BA. Current molecular models for NADPH oxidase regulation by Rac GTPase. Blood 2002;100:2692–2696.
[31] Rajesh HA, Chen H-Q, Veluthakal R, Silver RB, Li J, Li GD, Kowluru A. Mastoparan-induced insulin secretion from insulin-secreting ßTC3 and INS-1 cells: Evidence for its regulation by Rho subfamily of G proteins. Endocrinology 2003;144:4508–4518.
[32] Quinn MT, Evans T, Loetterle LR, Jesaitis AJ, Bokoch GM. Translocation of Rac correlates with NADPH oxidase activation. Evidence for equimolar translocation of oxidase components. J Biol Chem 1993;268:20983–20987.
[33] Lee M, You HJ, Cho SH, Woo CH, Yoo MH, Joe EH, Kim JH. Implication of the small GTPase Rac1 in the generation of reactive oxygen species in response to beta-amyloid in C6 astroglioma cells. Biochem J 2002;366:937–943.
[34] Lee SS, Woo CH, Chang JD, Kim JH. Roles of Rac and cytosolic phospholipase A_2 in the intracellular signalling in response to titanium particles. Cell Signal 2003;15:339–345.
[35] Bey EA, Xu B, Bhattacharjee A, Oldfield CM, Zhao X, Li Q, Subbulakshmi V, Feldman GM, Wientjes FB, Cathcart MK. Protein kinase C delta is required for p47phox phosphorylation and translocation in activated human monocytes. J Immunol 2004;173:5730–5738.