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Calcium overload is essential for the acceleration of staurosporine-induced cell death following neuronal differentiation in PC12 cells

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Abbreviations: NGF, nerve growth factor; PARP, poly ADP-ribose polymerase

Abstract

Differentiation of neuronal cells has been shown to accelerate stress-induced cell death, but the underlying mechanisms are not completely understood. Here, we find that early and sustained increase in cytosolic ($[Ca^{2+}]_c$) and mitochondrial Ca^{2+} levels ($[Ca^{2+}]_m$) is essential for the increased sensitivity to staurosporine-induced cell death following neuronal differentiation in PC12 cells. Consistently, pretreatment of differentiated PC12 cells with the intracellular Ca^{2+}-chelator EGTA-AM diminished staurosporine-induced PARP cleavage and cell death. Furthermore, Ca^{2+} overload and enhanced vulnerability to staurosporine in differentiated cells were prevented by Bcl-X_L overexpression. Our data reveal a new regulatory role for differentiation-dependent alteration of Ca^{2+} signaling in cell death in response to staurosporine.

Keywords: bcl-X protein; calcium; cell death; cell differentiation; PC12 cells; staurosporine

Introduction

Previously, it has been reported that PC12 cells differentiated into sympathetic neurons in response to nerve growth factor (NGF) are more sensitive to apoptotic stimuli, such as TNF-α and ethanol, than undifferentiated PC12 cells (Oberdoerster *et al*., 1999; Zhang *et al*., 2007). On the other hand, however, a carbonyl stressor (methylglyoxal) was shown to induce apoptosis more robustly in undifferentiated PC12 cells (Okouchi *et al*., 2005). These different reports suggest that mechanisms involved in death pathways are divided between neurotoxic factors and may be significantly influenced by cellular phenotypes.

In many cell types, alteration of intracellular Ca^{2+} homeostasis plays a pivotal role in initiating apoptosis (Park *et al*., 2002; Demaurex *et al*., 2003). Analysis of brain tissue from Alzheimer Disease (AD) patient showed that alteration of Ca^{2+} homoeostasis is associated with the neurofibrillary tangle-bearing neurons (Murray *et al*., 1992). Numerous findings have also suggested that perturbation of Ca^{2+} signaling contributes to many age-related neurodegenerative disorders, including: Parkinson's Disease (PD), Huntington's Disease (HD), ischemic stroke, and amyotrophic lateral sclerosis (Beal, 1998; Rodnitzky, 1999; Simpson, *et al*., 2002).

Increases in cytosolic ($[Ca^{2+}]_c$) or mitochondrial Ca^{2+} concentrations ($[Ca^{2+}]_m$) have been shown to mediate cell death in various cell types. For example, exposure of PC12 cells to staurosporine causes cytosolic and mitochondrial Ca^{2+} overload, which is an essential event for initiation of cell death (Kruman *et al*., 1998). Staurosporine, a broad spectrum protein kinase inhibitor, has been used to induce cell death in a wide range of cell types (Kabir *et al*., 2002; Witasp *et al*., 2005; Wang *et al*., 2007). Although the exact mechanism responsible for staurosporine-induced cell death is unknown, activation of caspases triggered by cytochrome c release from mitochondria into cytosol is required (Johansson *et al*., 2003).

We investigated if neuronal differentiation of PC12 cells by NGF accelerates staurosporine-induced cell death and if an increase in $[Ca^{2+}]_c$ is a contributing factor to the increased sensitivity to staurosporine-induced cell death following neuronal differentiation. Our data suggest that early and sustained increase in $[Ca^{2+}]_c$ is responsible for release of mitochondrial cytochrome c, caspase-3 activation, DNA fragmentation, and cell death in

neuronally differentiated PC12 cells exposed to staurosporine. Undifferentiated PC12 cells are more resistant to staurosporine-induced effects.

Results

Staurosporine induces cell death to a great extent in neuronally differentiated PC12 cells

Previously, neuronally differentiated PC12 cells appeared to be more sensitive to apoptotic stimuli, such as TNFα and ethanol, than undifferentiated cells (Oberdoerster and Rabin, 1999; Zhang *et al*., 2007). In the present study, we tested if NGF-induced neuronal differentiation in PC12 cells accelerates cell death in response to staurosporine, which has been used as a common inducer of cell death in almost all cell types. Staurosporine (at concentrations greater than 0.1 μM) induced the death of neuronally differentiated PC12 cells to a greater extent compared to undifferentiated PC12 cells (Figure 1A). The cell death provoked by 0.2 μM staurosporine progressed more rapidly over time in differentiated PC12 cells than in undifferentiated cells (Figure 1B). Therefore, our findings are in agreement with those recently reported by Zhang and colleagues (Oberdoerster and Rabin, 1999; Zhang *et al*., 2007), but not with those reported by Ekshyyan and Aw (Ekshyyan *et al*., 2005). Ekshyyan and Aw demonstrated that the transition of undifferentiated PC12 cells into differentiated cells affords protection against oxidative stress. While the discrepancy between these opposite observations has not been resolved, the alteration of survival or death signals during neuronal differentiation might be involved.

Staurosporine induces DNA fragmentation, caspase 3 activation, and release of mitochondrial cytochrome c into cytosol in neuronally differentiated PC12 cells

In an attempt to determine underlying mechanisms responsible for enhanced cell death by staurosporine in neuronally differentiated cells, we analyzed DNA fragmentation, a typical characteristic feature of apoptosis. As shown in Figure 2A, 0.2 μM staurosporine induced DNA fragmentation in neuronally differentiated cells, but not in undifferentiated cells. DNA fragmentation was accompanied by a sequential 85 kDa cleavage product of PARP, one of the targets associated with caspase activation (Figure 2B) (Lazebnik *et al*., 1994). Consistent with this, measurement of caspase 3 protease activity using the colorimetric substrate Ac-DEVD-*p*NA confirmed that staurosporine enhanced caspase 3 activation in the neuronally differentiated PC12 cells (Figure 2C). Since the mitochondrial cytochrome c released into the cytosol has been identified as an apoptosis initiation molecule (Desagher *et al*., 2000), we examined whether staurosporine accelerated the release of mitochondrial cytochrome c into the cytosol in neuronally differentiated PC12 cells. As shown in Figure 2D, 0.2 μM staurosporine caused a significant increase in cytosolic cytochrome c, and a decrease in mitochondrial cytochrome c, after treatment with staurosporine in neuronally differentiated cells, whereas the content of cytosolic and mitochondrial cytochrome c remained constant in undi-

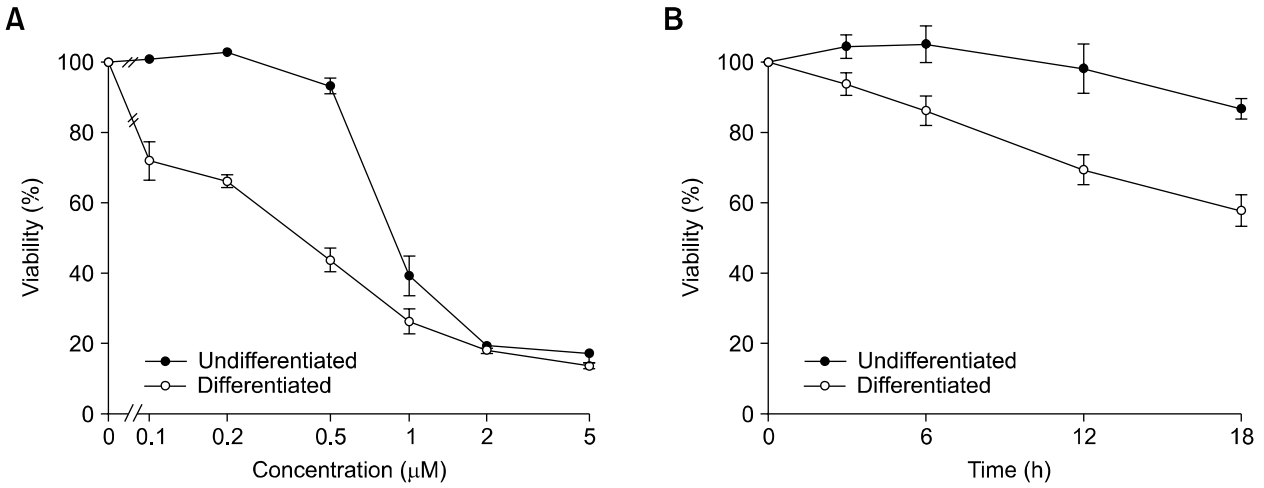

Figure 1. Neuronally differentiated PC12 cells exhibit increased sensitivity to staurosporine-induced cell death. Neuronally differentiated and undifferentiated PC12 cells were treated with indicated concentrations of staurosporine for 12 h (A) or treated with 0.2 μM staurosporine for indicated times (B). The viability of cells was determined by MTT assay. Results are presented as the means $\pm$ SD of four independent experiments.

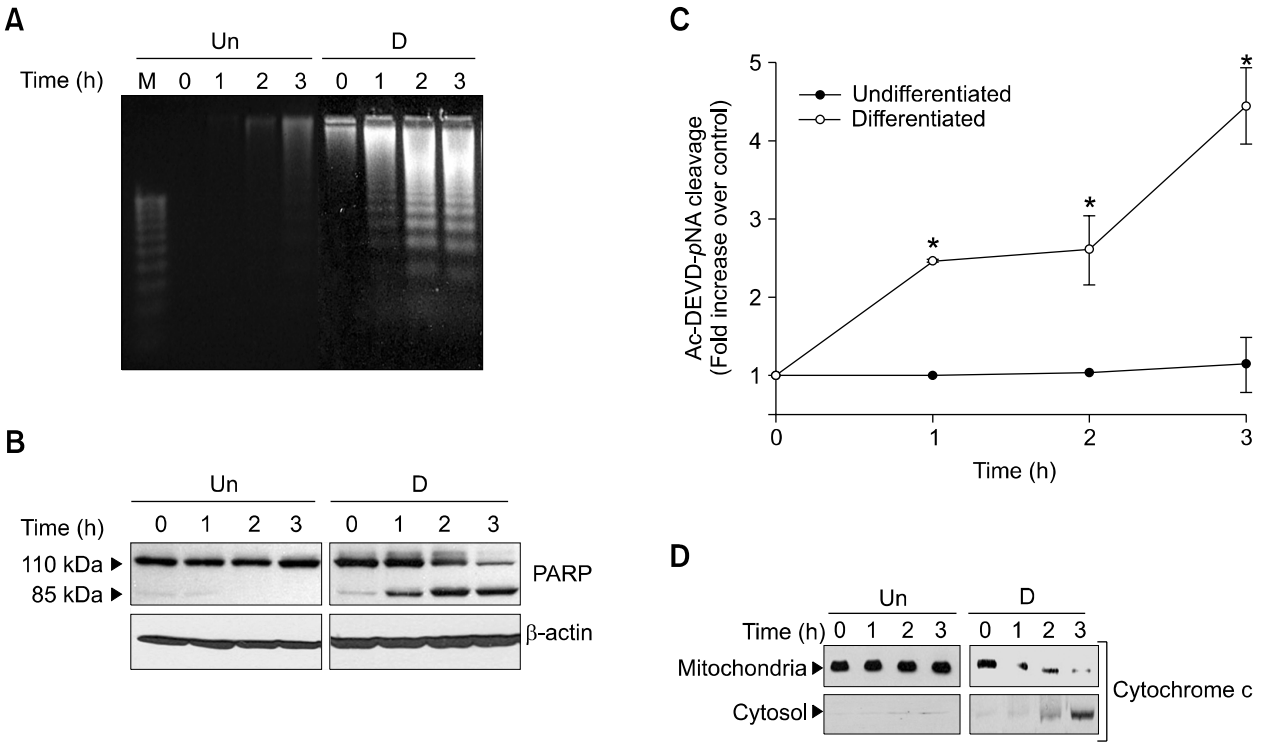

Figure 2. Staurosporine induces DNA fragmentation, caspase-3 activation, and cytochrome c release in neuronally differentiated PC12 cells. Neuronally differentiated and undifferentiated PC12 cells were treated with 0.2 μM staurosporine for the indicated times. (A) The fragmented DNA was analyzed by agarose gel electrophoresis. (B) Total cell extracts were immunoblotted with anti-PARP and anti-β-actin antibodies. (C) Caspase-3 activity was measured using a colorimetric substrate Ac-DEVD-*p*NA. Values are the means ± SD of three independent experiments. *Significantly different from undifferentiated cells ($P < 0.05$). (D) Cytoplasmic and mitochondrial fractions of extracts were immunoblotted with anti-cytochrome c antibody. D: differentiated PC12 cells, Un: undifferentiated PC12 cells.

fferentiated cells up to 3 h (Figure 2D).

Elevated $[Ca^{2+}]_m$ and $[Ca^{2+}]_c$ in differentiated PC12 cells are responsible for staurosporine-induced cell death

Several lines of evidence indicate that uncontrolled cytosolic or mitochondrial Ca^{2+} overload mediates staurosporine-induced cell death in neuronal cells (Prehn *et al*., 1997; Kruman *et al*., 1998, 1999). To determine if the alteration of Ca^{2+} homeostasis is essential for initiating differentiation-dependent, staurosporine-activated cell death signaling, we analyzed $[Ca^{2+}]_c$ and $[Ca^{2+}]_m$ in undifferentiated and neuronally differentiated PC12 cells.

Exposure of neuronally differentiated PC12 cells to 0.2 μM staurosporine resulted in early and sustained elevation of $[Ca^{2+}]_c$, whereas exposure of undifferentiated cells had little effect on $[Ca^{2+}]_c$ (Figure 3A). The $[Ca^{2+}]_c$ increase was completely prevented by 20 μM of the membrane permeable intracellular Ca^{2+} chelator EGTA-AM. It is well established that sustained overload of cytosolic Ca^{2+} causes enhanced accumulation of Ca^{2+} by the mitochondria, which sensitizes the cytochrome c release pathway (Szabadkai *et al*., 2004; Dong *et al*., 2006). Since staurosporine caused early and sustained increase in $[Ca^{2+}]_c$ in the present study, we speculated that Ca^{2+} might have accumulated in mitochondria. In this context, we assessed changes in $[Ca^{2+}]_m$ microscopically in living cells loaded with the mitochondrial Ca^{2+} indicator Rhod 2-AM, which detects free Ca^{2+} levels in the mitochondrial matrix. Following treatment with 0.2 μM staurosporine, we observed a significant increase in $[Ca^{2+}]_m$ in neuronally differentiated PC12 cells, but not in undifferentiated cells (Figure 3B). While we did not investigate the direct role of mitochondrial Ca^{2+} overload in cell death in this study, previous reports revealed that it is linked to mitochondrial membrane depolarization and ROS accumulation, which are believed to play a role in cell death (Hajnoczky *et al*., 2003).

We next analyzed whether chelating of intracellular Ca^{2+} could block the cleavage of PARP in neuronally differentiated PC12 cells (Figure 3C). Pretreatment of neuronally differentiated PC12 cells with intracellular Ca^{2+} chelators such as BAPTA-AM

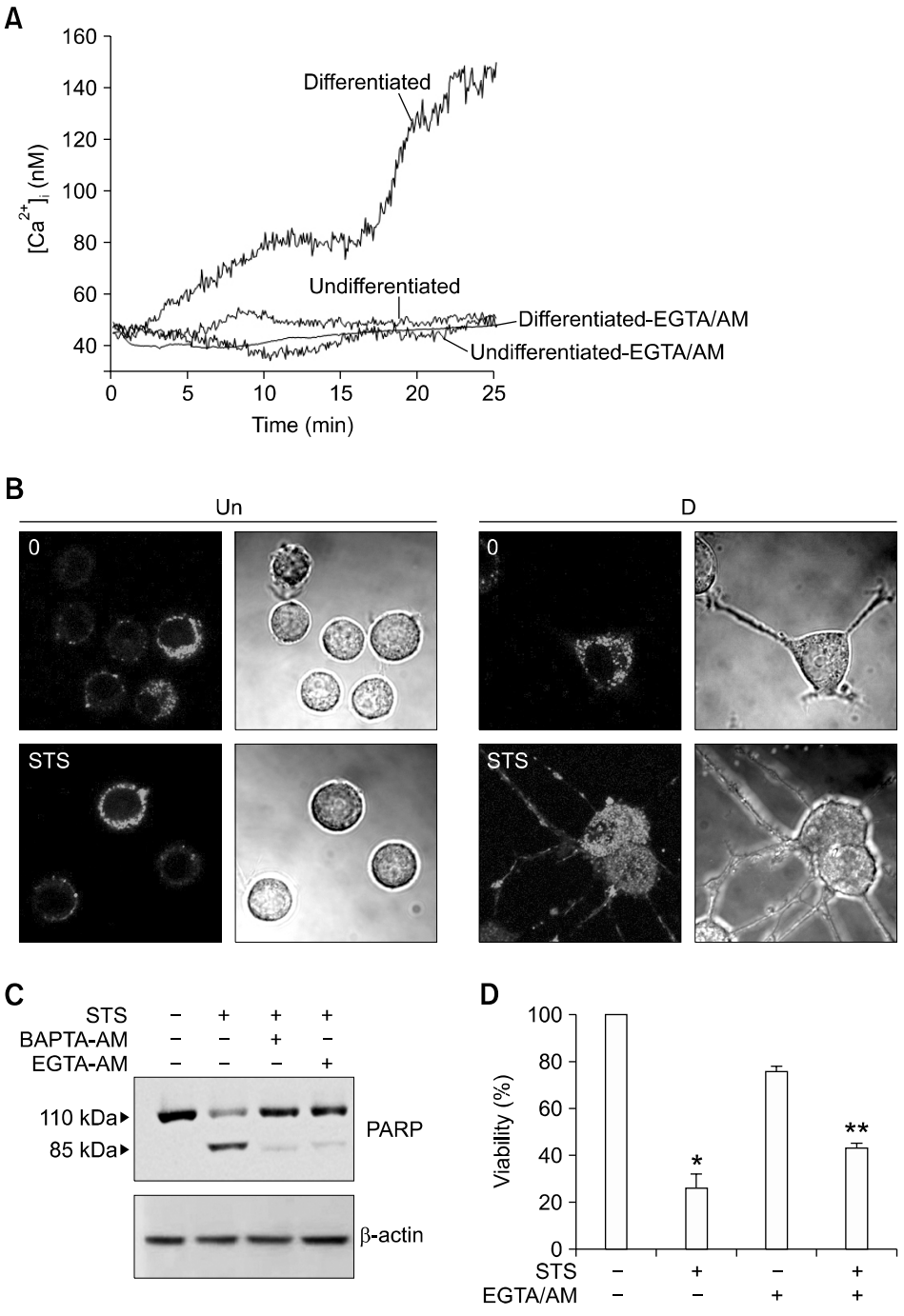

Figure 3. Staurosporine-induced increases in $[Ca^{2+}]_m$ and $[Ca^{2+}]_c$ are involved in the death of differentiated PC12 cells. (A) Changes in $[Ca^{2+}]_c$ were measured in fura-2 loaded neuronally differentiated and undifferentiated PC12 cells using ratiometric fluorescence recording techniques after the application of 0.2 μM staurosporine. In some experiments, cells were pretreated with 20 μM EGTA-AM for 10 min. (B) Changes in $[Ca^{2+}]_m$ were monitored in rhod-2 loaded neuronally differentiated (right) and undifferentiated PC12 cells (left), by confocal microscopy, after treatment with 0.2 μM staurosporine for 1 h. (C) Neuronally differentiated PC12 cells were treated with intracellular Ca^{2+} chelators (20 μM BAPTA-AM or 20 μM EGTA-AM) for 30 min followed by 0.2 μM staurosporine for 3 h. Cell lysates were then immunoblotted with anti-PARP and anti-β-actin antibodies. (D) Neuronally differentiated PC12 cells were treated with 0.2 μM staurosporine for 24 h either in the presence or absence of 10 μM EGTA-AM, and cell viability was measured by MTT assay. Values are the means ± SD of four independent experiments. *Significantly different from cells unexposed to staurosporine ($P < 0.05$). **Significantly different from cells exposed to staurosporine alone ($P < 0.05$).

and EGTA-AM inhibited the cleavage of PARP, suggesting that Ca^{2+} acts upstream of caspase 3 activation in the staurosporine-induced death process. Consistent with this, inhibition of $[Ca^{2+}]_c$ increase by EGTA-AM in differentiated cells attenuated the staurosporine-induced cell death (Figure 3D). These results indicate that neuronally differentiated PC12 cells are more sensitive to staurosporine-induced cell death than undifferentiated cells due, in part, to the enhanced increases in $[Ca^{2+}]_c$ in the differentiated cells.

Bcl-X_L prevents staurosporine-induced $[Ca^{2+}]_c$ increases and cell death

We next investigated if anti-apoptotic Bcl-X_L antagonizes staurosporine-induced cell death in differentiated PC12 cells as reported previously (Boise

et al., 1993; Gonzalez-Garcia *et al*., 1994). Bcl-X_L is an anti-apoptotic member of the Bcl-2 family, which is localized to the membranes of nuclear envelope, ER, and mitochondria (Lithgow *et al*., 1994). While the mechanism of Bcl-X_L is still debated, multiple mechanisms are believed to be involved in the protection of cells from apoptosis.

As shown in Figure 4A, 0.2 μM staurosporine-induced neuronal cell death was largely prevented in Bcl-X_L overexpressing PC12 cells. The inhibitory effect of Bcl-X_L was also observed on DNA fragmentation and the PARP cleavage pattern (Figure 4B and C).

Since our results indicate that an increase in $[Ca^{2+}]_c$ is an early event in the staurosporine-induced apoptotic process, we next analyzed whether Bcl-X_L could inhibit the $[Ca^{2+}]_c$ increase in neuronally differentiated PC12 cells. As shown in Figure 4D, treatment of Bcl-X_L overexpressing stable cells with 0.2 μM staurosporine prevented the increase in $[Ca^{2+}]_c$, confirming that the anti-apoptotic action of Bcl-X_L is accompanied by the inhibition of $[Ca^{2+}]_c$ increase.

Discussion

PC12 cells differentiate into neuronal cells with neurite extensions in response to NGF (Hatayama *et al*., 1997). Several controversial reports show that neurotoxic effect was different between undifferentiated and differentiated phenotypes of PC12 cells (Oberdoerster and Rabin, 1999; Okouchi *et al*., 2005; Zhang *et al*., 2007).

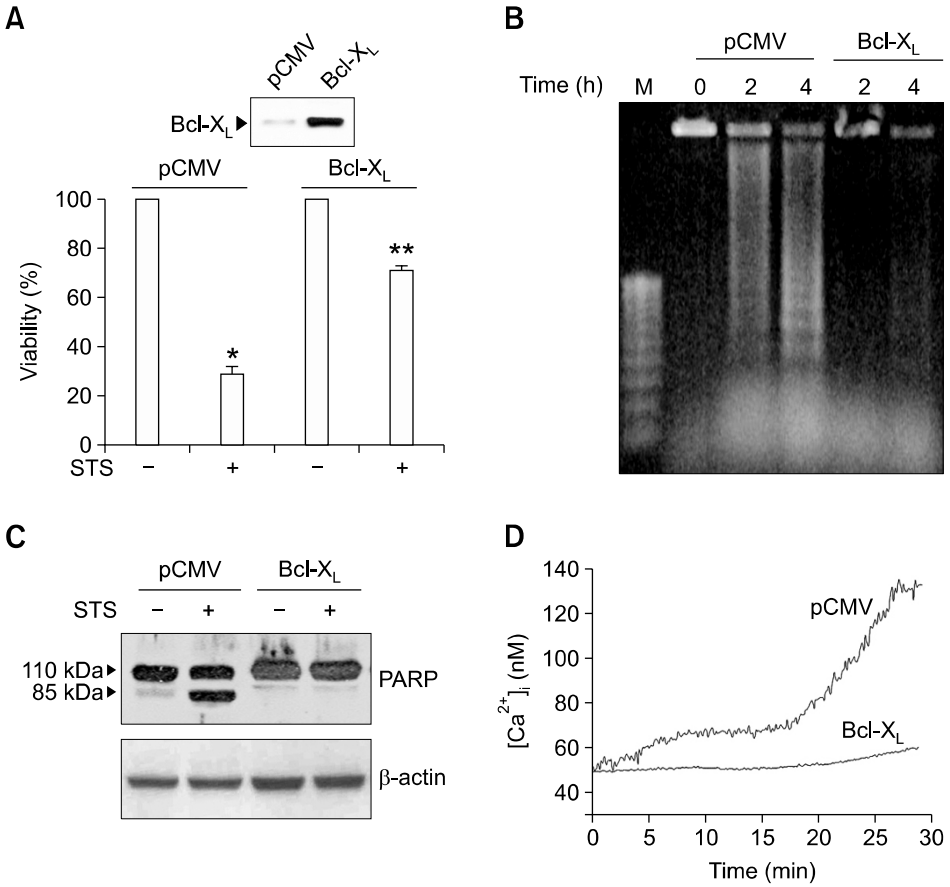

Figure 4. Overexpression of Bcl-X_L prevents DNA fragmentation, PARP cleavage, $[Ca^{2+}]_c$ increase, and cell death in neuronally differentiated PC12 cells. (A) Bcl-X_L overexpressing (Bcl-X_L) and control (pCMV) PC12 cells were differentiated and treated with 0.2 μM staurosporine for 24 h, and cell viability was measured by MTT assay. Values are the means ± SD of four independent experiments. *Significantly different from cells unexposed to staurosporine ($P < 0.05$). **Significantly different from pCMV-transfected cells exposed to staurosporine ($P < 0.05$). (B) Bcl-X_L overexpressing (Bcl-X_L) and control (pCMV) PC12 cells were differentiated and treated with 0.2 μM staurosporine for indicated times and the fragmented DNA was analyzed by agarose gel electrophoresis. (C) Bcl-X_L overexpressing (Bcl-X_L) and control (pCMV) PC12 cells were differentiated and treated with 0.2 μM staurosporine for 6 h, and total cell extracts were immunoblotted with anti-PARP and anti-β-actin antibodies. (D) Changes in $[Ca^{2+}]_c$ were measured in fura-2 loaded, neuronally differentiated Bcl-X_L overexpressing cells (Bcl-X_L) and control (pCMV) using ratiometric fluorescence recording techniques after the application of 0.2 μM staurosporine.

In this study, we analyzed the response of neuronally differentiated and undifferentiated PC12 cells to staurosporine to elucidate whether cellular state determines apoptotic sensitization and found that differentiated neuronal cells respond more sensitively to staurosporine than undifferentiated cells. Our results provide evidence that the alteration of Ca^{2+} homeostasis following NGF-induced differentiation is directly correlated with the acceleration of staurosporine-induced apoptotic commitment in PC12 cells; intracellular Ca^{2+} overload in response to staurosporine is evident in differentiated PC12 cells compared with undifferentiated cells. To our knowledge, this is the first report to highlight the role of differentiation-dependent alteration of Ca^{2+} signaling in cell death in response to staurosporine.

At present, it is unclear why the potency and efficacy of staurosporine to cause increase of $[Ca^{2+}]_c$ and $[Ca^{2+}]_m$ differs between undifferentiated and differentiated neuronal cells. We speculate that the expression of proteins involved in the regulation of intracellular Ca^{2+} homeostasis, including Ca^{2+} channels and ATPase on endoplasmic reticulum or plasma membrane, is modulated during the neuronal differentiation process. In supports of this, several lines of reports suggested that NGF induces expression of several types of ion channels (Usowicz *et al*., 1990; Furukawa *et al*., 1993; Lewis *et al*., 1993; Jimenez *et al*., 2001). For example, increased expression of Na^+ and Ca^{2+} channels in PC12 cells, and ryanodine receptor isoform 2 (RyR2) in rat chromaffin cells by the NGF treatment (Furukawa *et al*., 1993; Jimenez and Hernandez-Cruz, 2001). Thus, alteration of voltage-gated Ca^{2+} influx and Ca^{2+} release from intracellular stores might cause the acceleration of staurosporine-induced apoptotic process.

Consistent with actions of staurosporine on alterations of Ca^{2+} homeostasis following differentiation of PC12 cells, we found that Bcl-X_L prevented staurosporine-induced neuronal cell death. Bcl-X_L, a member of anti-apoptotic Bcl-2 subfamily, has been shown to interfere with Ca^{2+}-mediated apoptotic signals by inhibiting Ca^{2+} release from ER. In the present study, we showed that the anti-apoptotic effect of Bcl-X_L was accompanied by the inhibition of $[Ca^{2+}]_c$ increases in differentiated PC12 cells exposed to staurosporine. These results are in good agreement with those obtained by Wang and colleagues (Wang *et al*., 2007). They found that Bcl-X_L blocked cytochrome c release and caspase-3 activation in response to staurosporine in rat hepatocytes. Furthermore, Li and colleagues reported that less Ca^{2+} was released from the ER in Bcl-X_L expressing cells in response to apoptotic stimuli due to down-regulation of IP_3 receptors (Li *et al*., 2002).

In fact, we observed that despite the complete chealation of intracellular Ca^{2+} and blockage of caspase 3 activity by EGTA-AM, staurosporine-induced cell death was not completely blocked by EGTA-AM in differentiated cells, raising the possibility that staurosporine could activate death pathway through a mechanism that is independent of Ca^{2+}. Although increase in $[Ca^{2+}]_c$ is not the only death mechanism induced by staurosporine, it is likely that acceleration of death in differentiated PC12 cells is primarily mediated through the Ca^{2+}-dependent pathway.

Collectively, our findings suggest that increased sensitivity to staurosporine-induced death in neuroanlly differentiated PC12 cells might be caused by the alteration of Ca^{2+} signaling during the differentiation process.

Methods

Cells and reagents

PC 12 cells were obtained from ATCC (Manassas, VA), and PC12 cells overexpressing Bcl-X_L were kindly provided by Dr. Y. J. Oh (Yonsei University, Seoul, Korea). BAPTA-AM, EGTA-AM, anti-poly-(ADP-ribose) polymerase (PARP) antibody, and acetyl-Asp-Glu-Val-Asp-*p*-nitroanilide (Ac-DEVD-*p*NA) were purchased from Calbiochem (San Diego, CA). Anti-cytochrome c antibody was purchased from Pharmingen (San Diego, CA). Fura-2 AM and Rhod-2 AM were purchased from Molecular Probes (Eugene, OR). NGF was purchased from Alomone labs (Jerusalem, Israel). Other reagents, including staurosporine, were obtained from Sigma (St. Louis, MO).

Cell culture

Cultures were maintained in DMEM (Life technologies, Inc.) supplemented with 10% heat-inactivated horse serum and 5% FBS (Life technologies, Inc.). To obtain neuronally differentiated PC12 cultures, cells were grown on collagen coated plates (10 μg/ml; Upstate Biotechnology, Lake Pacid, NY) supplemented with 2% heat-inactivated horse serum, 1% FBS, and 50 ng/ml NGF for seven days. The medium, including NGF, was replaced every two days. Cultures were maintained at 37°C in a humidified, 5% CO_2 incubator. In differentiated PC12 cells, all experiments were performed in the presence of NGF to exclude the possibility of NGF-deprived cell death signaling pathways.

MTT assay

After each indicated treatment, cells were incubated with MTT at a final concentration of 1 mg/ml for 1 h at 37°C, followed by lysis in solubilizing solution (50% dimethylformamide and 20% SDS, pH 4.8) for 24 h. The absorption value was determined at 570 nm and viability was

determined as percent survival relative to untreated control.

Immunoblot analysis

Cells were lysed in buffer containing 10 mM Tris-Cl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 0.5% Nonidet P-40, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 1 μg/ml leupeptin, and 1 μg/ml aprotinin. Equal amounts of proteins were separated on 10% SDS-polyacrylamide gels and subsequently transferred to nitrocellulose membranes. Specific immunodetection was carried out by incubation with indicated antibodies followed by peroxidase-conjugated anti-mouse or anti-rabbit IgG antibody. Blots were evaluated using an ECL detection system.

Measurement of $[Ca^{2+}]_c$ and $[Ca^{2+}]_m$

For $[Ca^{2+}]_c$ measurements, PC12 cells were loaded with 4 μM fura-2 AM at 37°C in a 5% CO_2 incubator for 20 min in a HCO_3^--buffered solution containing [110 mM NaCl, 4.5 mM KCl, 1 mM NaH_2PO_4, 1 mM Mg_SO_4, 1.5 mM $CaCl_2$, 5 mM HEPES-Na, 5 mM HEPES free acid, 25 mM $NaHCO_3$ and 10 mM D-glucose (pH 7.4)]. Cells were then rinsed twice and incubated in the HCO_3^--buffered solution for at least 20 min before use. $[Ca^{2+}]_c$ was measured on the stage of an inverted microscope (Nikon, Tokyo, Japan) by spectrofluorometry (Photon Technology International, Brunswick, NJ), while cells were superfused at a constant perfusion rate of 2 ml/min with the HCO_3^--buffered solution equilibrated with 95% O_2, 5% CO_2 to maintain pH 7.4. All experiments were performed at 37°C. The excitation wavelength was alternated between 340 and 380 nm and the emission fluorescence was recorded at 510 nm. $[Ca^{2+}]_c$ values were calculated using the equation described by Grynkiewicz (Grynkiewicz *et al*., 1985). Relative $[Ca^{2+}]_m$ was measured with the fluorescent probe rhod 2-AM following methods described previously (Hoth *et al*., 1997). In brief, cells were loaded with 4 μM rhod-2 AM for 60 min. The residual cytosolic fraction of the dye was eliminated when cells were kept in culture for an additional 6 h after loading, whereas the mitochondrial dye fluorescence was retained. Cellular fluorescence was imaged using a confocal microscope with excitation at 514 nm and emisson at 535 nm.

DNA fragmentation analysis

Cells were lysed in 0.05% Triton X-100, 20 mM EDTA, and 10 mM Tris-Cl (pH. 8.0) for 30 min and the fragmented DNA was precipitated with ethanol. The precipitates were then resuspended in TE buffer and electrophoresed on 1.5% agarose gels.

Caspase 3 assay

Cells were lysed in buffer containing 1 mM KCl, 1.5 mM MgCl, 1 mM DTT, 1 mM PMSF, 5 μg/ml leupeptin, 2 μg/ml aprotinin and 10% glycerol. 20 μg of each protein were added to reaction buffer [25 mM HEPES (pH 7.4), 10 mM DTT, 10% sucrose, 0.1% CHAPS] containing 40 μM Ac-DEVD-*p*NA, a colorimetric substrate for caspase-3 protease activity (Stefanis *et al*., 1996), and incubated at 37°C for 30 min. DEVD-*p*NA cleavage was measured at 405 nm.

Subcellular fractionation

Subcellular fractionation was performed according to previously reported methods (Gross *et al*., 1999). Briefly, PC12 cells were homogenized in five volumes of extraction buffer containing 220 mM mannitol, 68 mM sucrose, 50 mM PIPES-KOH (pH 7.4), 50 mM KCl, 5 mM EGTA, 2 mM $MgCl_2$, 1 mM DTT and 1 mM PMSF. Cells were then spun at 400 × *g* for 10 min at 4°C to separate out nuclei and unbroken cells. Supernatant was centrifuged at 10,000 × *g* for 10 min at 4°C to collect the mitochondrial-enriched pellet. The new supernatant was then spun at 100,000 × *g* for 30 min at 4°C to separate the light membrane ER-enriched pellet (not used in these experiments) from the supernatant (containing the cytosol).

Statistical analysis

Results are presented as the means ± SD of three or four independent experiments. When comparing two groups, an unpaired Student's *t*-test was used to address differences. *P*-values less than 0.05 were considered significant.

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