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Resveratrol protects SH-SY5Y neuroblastoma cells from apoptosis induced by dopamine

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Abbreviations: DA, dopamine; MMP, mitochondrial membrane potential; PI, propidium iodide; PS, phosphatidyl serine

Abstract

Dopamine (DA) is an oxidant that may contribute to the degeneration of dopaminergic neurons. The present study demonstrates that DA-induced cytotoxicity in human-derived neurotypic cells, SH-SY5Y, is prevented by resveratrol, one of the major antioxidative constituents found in the skin of grapes. SH-SY5Y cells, a neuroblastoma cell line, treated with DA at 300 and 500 μM for 24 h underwent apoptotic death as determined by characteristic morphological features, including nuclear condensation, and loss of mitochondrial membrane potential (MMP). Flow cytometric analysis using Annexin V showed that DA can induce significant and severe apoptosis. Exposure to resveratrol (5 μM) for 1 h prior to the DA treatment attenuated DA-induced cytotoxicity, and rescued the loss of MMP. To investigate the apoptotic signaling pathways relevant to the restoration of DA-induced apoptosis by resveratrol, we carried out quantitative analysis of Bcl-2, caspase-3, and cleaved poly ADP-ribose polymerase (PARP) by immunoblot analysis. Resveratrol pretreatment led to a decrease in cleavage of PARP, an increase in the Bcl-2 protein, and activation of caspase-3. These results suggest that DA may be a potential oxidant of neuronal cells at biologically relevant concentrations. Resveratrol may protect SH-SY5Y cells against this cytotoxicity, reducing intracellular oxidative stress through canonical signal pathways of apoptosis and may be of biological importance in the prevention of a dopaminergic neurodegenerative disorder such as Parkinson disease.

Keywords: antioxidant; apoptosis; dopamine; neuroblastoma; neurodegenerative diseases; resveratrol

Introduction

Parkinson's disease (PD) is a common neurodegenerative disease, characterized by a selective loss of dopaminergic neurons in the substantia nigra. Many factors are speculated to operate in the mechanism of cell death of nigrostriatal dopaminergic neurons in PD, including oxidative stress and cytotoxicity of reactive oxygen species (ROS), disturbances of intracellular calcium homeostasis, exogenous and endogenous toxins, and mitochondrial dysfunction. An endogenous neurotransmitter dopamine (DA) is thought to be a major source of oxidative stress to these neural cells (Olanow, 1993; Offen *et al*., 1997). DA contains an unstable catechol moiety, and it can oxidize spontaneously to form ROS, free radicals, and quinones (Cohen and Heikkila, 1974; Graham, 1978; Hastings, 1995). In the human substantia nigra, the DA oxidation products may further polymerize to form another neurotoxin, neuromelanin (Jellinger *et al*., 1993). These oxidation products can damage cellular components such as lipids, proteins, and DNA (Halliwell, 1992).

One of the plausible ways to prevent the cell death induced by oxidative stress is dietary or pharmacological intake of antioxidants. Lai and Yu (1997) showed that DA-induced apoptosis is protected by some antioxidants such as glutathione and N-acetyl-$_L$-cysteine. One family of naturally occurring compounds possessing free radical scavenging properties is the polyphenols. It is found in fruit, vegetables and plant-derived beverages and may have important roles as dietary components via cytoprotective actions in many organs (Youdim and Joseph, 2001). Resveratrol (3,5,4'-tihydroxy-*trans*-stilbene), a

polyphenolic phytoalexin found in the skin of grapes, has been reported to possess a wide range of biological and pharmacological activities including antioxidant, anti-inflammatory, antimutagenic, and anticarcinogenic effects (Jang *et al*., 1997; Soleas *et al*., 1997; Surh, 1999). In previous studies, antioxidant properties of resveratrol attenuated hippocampal cell death and intracellular ROS formation (Bastianetto *et al*., 2000). Neuroprotective activities against excitotoxic brain damage (Virgili and Contestabile, 2000) and anti-apoptotic effects by caspase activation (Nicolini *et al*., 2001) suggest a putative neuroprotective action for resveratrol.

In the present study, we have examined the possible protective effect of resveratrol against apoptosis induced by DA in human derived neurotypic cell, SH-SY5Y. Our results indicate that DA indeed triggers apoptosis and this deleterious effect can be attenuated by resveratrol.

Materials and Methods

Materials

MEM, FBS and penicillin and streptomycin were obtained from Gibco BRL (Grand Island, NY). Resveratrol, dopamine, DMSO, Hoechst 33254, propidium iodide (PI) were obtained from Sigma (St. Louis, MO). DA and resveratrol were dissolved in sterilized H_2O and in DMSO, respectively. Annexin V was from BD Biosciences (Palo Alto, CA). Monoclonal antibodies against caspase-3, Bcl-2, cleavage form of PARP were obtained from Cell Signaling Technology (Danvers, MA). Bicinchoninic acid protein assay kit and ECL chemiluminescence system were obtained from Pierce (Rockford, IL).

Cell culture

Catecholaminergic neuroblastoma SH-SYSY cells were grown in MEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were maintained in a humidified atmosphere of 5% CO_2, at 37°C. The cells were plated at an appropriate density according to each experimental scale.

Cell viability assay

Cell viability was determined using Cell Titer 96 Aqueous Non-Radioactive Cell Proliferation Assay, available from Promega Corp (Madison, WI). MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazo-lium inner salt) conversion assay was performed according to the protocol supplied by the manufacturer. In brief, a mixture of MTS and PMS (phenazine methosulfate) was added to the treated SH-SY5Y cells cultured in 96-well plates and incubated in the CO_2 incubator for 3 h. Plates were read with a microplate reader at a wavelength of 490 nm. Results were expressed as the percentage of MTS reduction, assuming that the absorbance of control cells was 100%. For each experiment, all treatments were performed in triplicate wells.

Nuclear staining for assessment of apoptosis

SH-SY5Y cells were stained with the chromatin-specific dye Hoechst 33324 (Molecular Probes, Eugene, OR). The SH-SY5Y cells on the chamber culture slide were incubated for 2 days, and then treated with or without the resveratrol and DA at different concentrations. SH-SY5Y cells were fixed for 10 min with 3.5% paraformaldehyde in PBS at room temperature. After twice rinses with PBS, the cells were stained with 10 μg/ml Hoechst 33324 in PBS for 10 min at 37°C. Mounted slides were analyzed under a fluorescence microscope (Axiovert 135, ZEISS, Germany) using a mercury lamp through a 360-370 nm bandpass filter. Light emitted from Hoechst dye was collected through a 420 nm longpass filter.

Measurement of mitochondrial membrane potential

The level of mitochondrial membrane potential (MMP) was determined by flow cytometry and the fluorescent dye rhodamine 123. The rhodamine 123 data were recorded using an FL-1 photomultiplier. Sample data (10,000 cells) were used to prepare histograms of the Cell Quest data analysis program (Becton Dickinson).

Flow cytometric analysis using Annexin V and PI

SH-SY5Y cells were centrifuged to remove the medium, washed with PBS and stained with Annexin V-FITC and PI in binding buffer (10 mM Hepes, 140 mM NaCl, 2.5 mM $CaCl_2$). Ten thousand events were collected on each sample. Stained cells were analyzed using a FACScalibur (Becton Dickinson, Mountain View, CA) in the FL1-H and FL2-H channels.

Immunoblot analysis

Blotting was performed essentially as described previously (Schulz *et al*., 1999). The cells treated with or without resveratrol and DA, were washed once with ice-cold PBS and then lysed using ice-cold RIPA buffer and protease inhibitor cocktail (Sigma) at 4°C for 30 min. Cell lysates were centrifuged at 13,000 rpm for 25 min, and the protein concentrations were determined by the bicinchoninic acid method (Pierce, Rockford, IL) using BSA as

standard. The protein lysates were separated by 12% SDS-PAGE and transferred to nitrocellulose membrane (Bio-Rad, CA). Protein expression was detected using primary antibodies and secondary antibodies conjugated with HRP.

Ressults

Resveratrol protected SH-SY5Y cells against DA-induced cytotoxicity

DA concentrations from 0 to 500 μM were tested and demonstrated that DA is capable of induce metabolic interruption in SH-SY5Y cells in a dose range of 300-500 μM (Figure 1A). DA decreased cell viability concentration over time (Figure 1B). Prior studies have shown that resveratrol at up to 10 μM slightly promotes cell proliferation, while higher concentration resulted in significant apoptosis (Stervbo *et al*., 2006). We now found that 5 μM resveratrol slightly but significantly increased cell proliferation and DA-induced apoptotic effects were markedly attenuated by the co-presence of 3 or 5 μM resveratrol (Figure 1C).

Resveratrol attenuated DA-induced apoptotic cell death

SH-SY5Y cells treated for 24 h with DA at concentrations of 300 and 500 μM exhibited morphological alterations such as cell shrinkage and membrane blebbing that are normally associated with the occurrence of apoptotic cell death. Compared to the normal SH-SY5Y cells (Figure 2A), cells treated with DA round up, detached from the bottom, and aggregated as assessed by phase-contrast microscopy (Figure 2B and D). Resveratrol pretreatment mitigated these pathologic morphological features (Figure 2C and E). The presence of apoptotic cells was further confirmed by measurement of nuclear

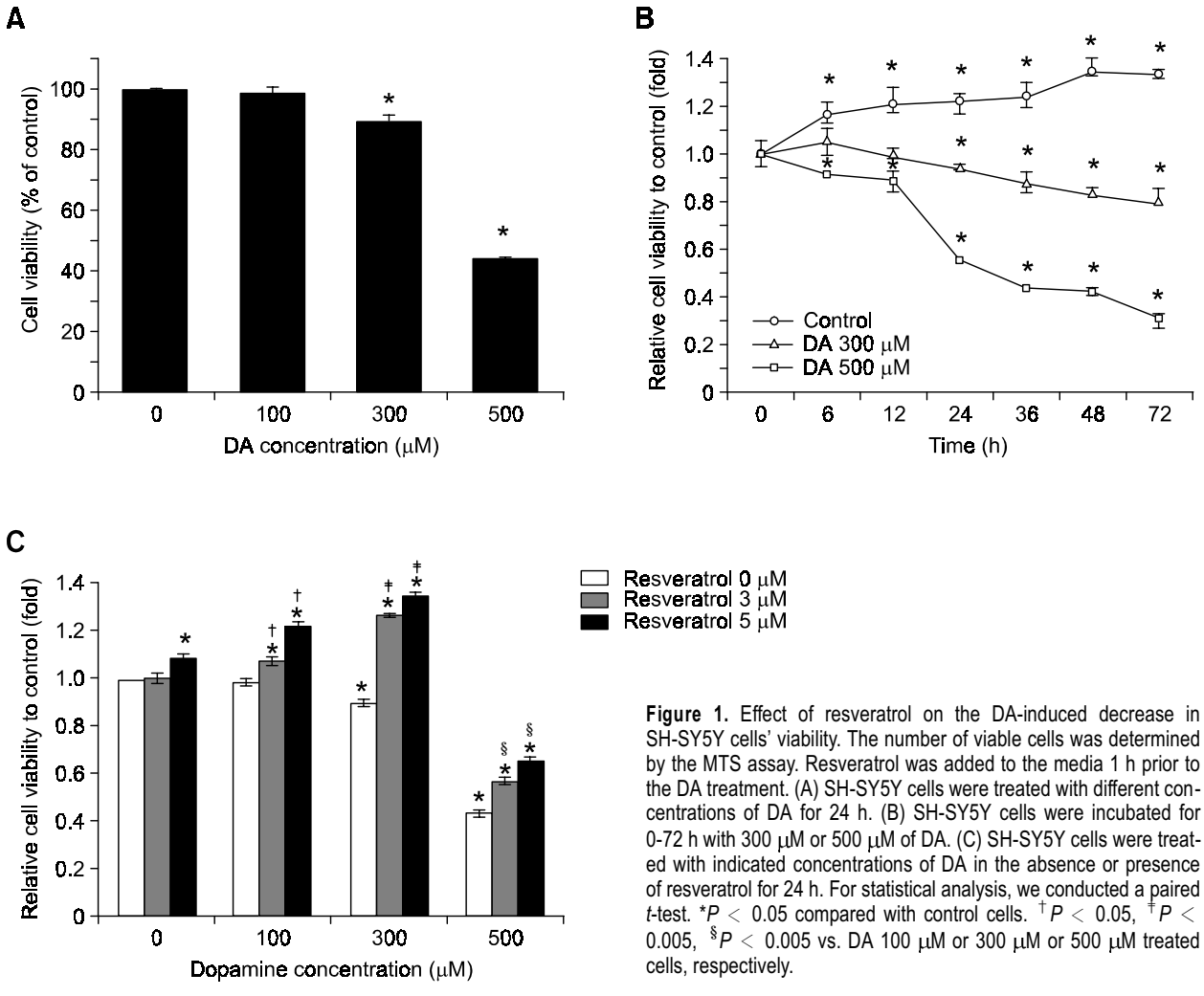

Figure 1. Effect of resveratrol on the DA-induced decrease in SH-SY5Y cells' viability. The number of viable cells was determined by the MTS assay. Resveratrol was added to the media 1 h prior to the DA treatment. (A) SH-SY5Y cells were treated with different concentrations of DA for 24 h. (B) SH-SY5Y cells were incubated for 0-72 h with 300 μM or 500 μM of DA. (C) SH-SY5Y cells were treated with indicated concentrations of DA in the absence or presence of resveratrol for 24 h. For statistical analysis, we conducted a paired *t*-test. $^{*}P < 0.05$ compared with control cells. $^{\dagger}P < 0.05$, $^{\ddagger}P < 0.005$, $^{\S}P < 0.005$ vs. DA 100 μM or 300 μM or 500 μM treated cells, respectively.

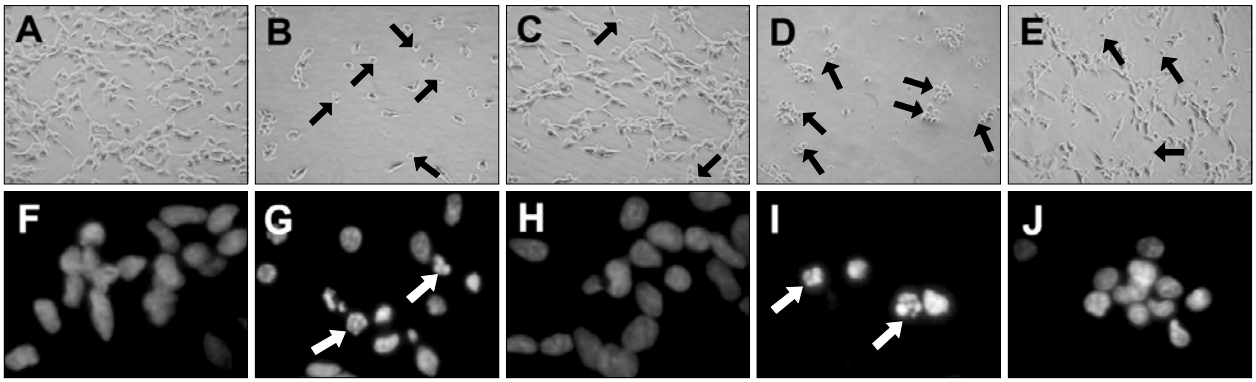

Figure 2. Microscopic analysis of resveratrol on DA-induced cell death. The figures show the phase-contrast photographs of SH-SY5Y cells after DA treatment in the absence or presence of resveratrol. The black arrows indicate typical apoptotic cells with round shape. Nuclear condensation was determined by Hoechst 33342 staining. The white arrows indicate apoptotic cells demonstrating partially condensed nuclei: (A, F) No treatment; (B, G) DA 300 μM; (C, H) DA 300 μM + resveratrol 5 μM; (D, I) DA 500 μM; (E, J) DA 500 μM + resveratrol 5 μM.

condensation using Hoechst 33342 (Hanrott *et al.*, 2006). Treatment with 300 and 500 μM DA significantly increased the proportion of condensed nuclei (Figure 2G and I). This incidence of cells with nuclear condensation was significantly reduced in DA-treated cells by 5 μM resveratrol treatment (Figure 2H and J). In addition, DNA fragmentation analysis was carried out, suggesting that DA-induced apoptosis was significantly reversed by resveratrol (data not shown).

Resveratrol had a protective effect on DA-induced dissipation of the mitochondrial membrane potential

The Bcl-2 family has a regulatory role in mitochondrial permeability transition pore opening and has an anti-apoptotic property (Crompton, 2000). Upon mitochondrial permeability transition pore opening, mitochondria lose their membrane potential across the inner membrane. Disruption of the MMP has been recognized in DA-induced apoptosis (Tang *et al.*, 2005a). To examine if DA-induced apoptosis and its rescue by resveratrol involve an MMP pathway in SH-SY5Y cells, measurement of MMP was carried out using rhodamine 123 (Tang *et al.*, 2005b). Preconditioning with DA alone induced the rapid depolarization of the MMP. In 300 and 500 μM DA-conditioned media, the degrees of depolarization were 65.1% and 74.5%, respectively (Figure 3B and D). However, preconditioning with resveratrol at 5 μM had protective effects with 43.5% and 55.6% of MMP at 300 and 500 μM DA, respectively ($P < 0.005$, each when compared to without resveratrol). These results indicate that DA-induced dissipation of the MMP can be effectively blocked by the pretreatment with resveratrol.

DA-induced cell death is attenuated in resveratrol-treated cells

To quantify cell death induced by DA, we have utilized flow cytometric analysis of externalization of phosphatidyl serine using Annexin V/PI. As shown in Figure 4, cells stained negative for both Annexin V and PI (PS^-/PI^-) were live cells as shown in the lower left quadrant (R3). Annexin V positive and PI-negative (PS^+/PI^-) stained cells undergo early stages of apoptosis, where the plasma membrane are still intact and exclude PI (lower right quadrant, R4). In late stages of apoptosis, dying cells can no longer exclude PI and the upper right region (R2) displayed both Annexin V-positive and PI-positive (PS^+/PI^+). PI positive and Annexin V-negative (PS^-/PI^+) stained cells in the upper left region (R1) were necrotic cells. Our data confirms that DA was capable of inducing cell death in SH-SY5Y cells. The increases of cell death over control in 300 μM DA treated cells were 6.6% in R2, and 24.1% in R4, respectively. Exposure to DA resulted in a significant increase in cell death (30.7%, $P < 0.01$ vs control) in SH-SY5Y cells. Similarly, 500 μM DA-treated cells also exhibited a marked increase in cell death (54.1%, $P < 0.01$ vs control). In contrast, resveratrol-pretreated SH-SY5Y cells showed significant resistance to DA-induced cytotoxicity because approximate 19.4% and 31.4% of the cells were affected, respectively (Figure 4, $P < 0.01$ vs. DA-only treated cells).

Resveratrol influenced the DA-induced apoptotic signaling pathway

To gain further insight into the way in which resveratrol is neuroprotective, we studied its effect on the possible caspases activated during the process of

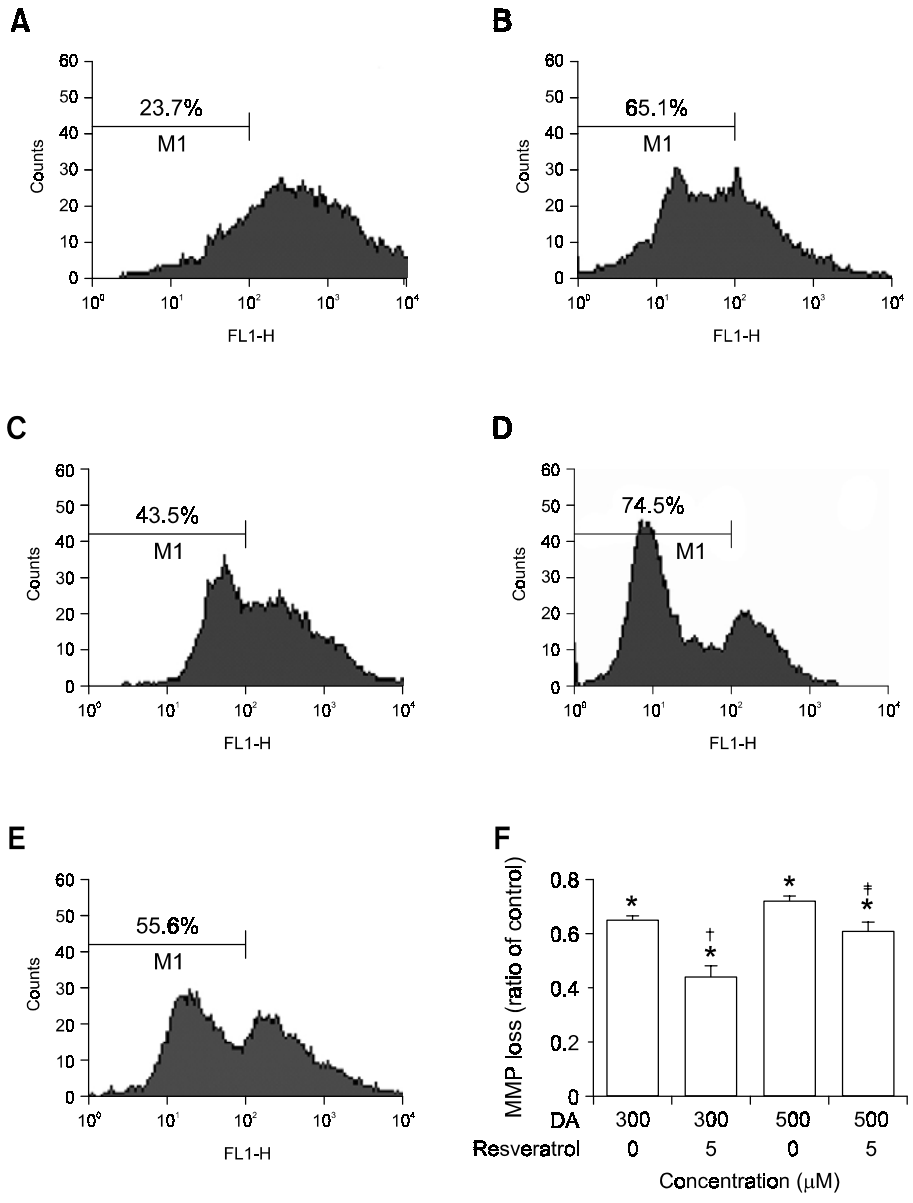

Figure 3. Effect of resveratrol preconditioning on DA-induced dissipation of mitochondrial membrane potential. MMP alteration was measured by flow cytometry using rhodamine 123 staining. The number in M1 indicates the percentage of cells with a reduced MMP level. SH-SY5Y cells were incubated in drug-free medium (A) or medium containing 300 μM or 500 μM DA (B, D); or cells were preincubated with 5 μM resveratrol and then exposed to DA for 24 h (C, E). (F) A bar graph summary of the averages of rhodamine 123 data collected from three independent flow cytometry experiments is shown. Bars represent the standard error of the mean. For statistical analysis, we conducted a Student paired *t*-test. $^{*}P < 0.001$ compared with control cells. $^{\dagger}P < 0.005$, $^{\ddagger}P < 0.005$ vs. DA 300 μM or 500 μM treated cells.

apoptosis. Caspase-3 is activated by multiple proteolytic cleavage of its 32 kDa precursor form to generate an enzymatically active p12/p17 complex, which has been used to monitor the activation of caspase-3 (Nicholson *et al.*, 1995). Treatment of SH-SY5Y cells with 300 and 500 μM DA induced the cleavage of caspase-3, demonstrated by the appearance of p17 fragments detected by an antibody for the cleaved form of caspase-3 and full-length caspase-3 (Figure 5). The increase in caspase-3 cleavage was associated with increased poly ADP-ribose polymerase (PARP) cleavage, a down stream substrate of caspase-3, and the extent of DA-induced PARP cleavage was also rescued by resveratrol (Figure 5).

We also examined the expression of Bcl-2 protein. Treatment of SH-SY5Y cells with DA decreased the expression of Bcl-2, which was reversed by resveratrol pretreatment (Figure 5). The partial restoration of Bcl-2 by resveratrol implies the possible involvement of other antiapoptotic proteins in regulating the death and survival in SH-SY5Y cells. These results suggested that resveratrol treatment shifted the balance between positive and negative regulators of apoptosis towards cell survival through canonical apoptosis signaling pathways.

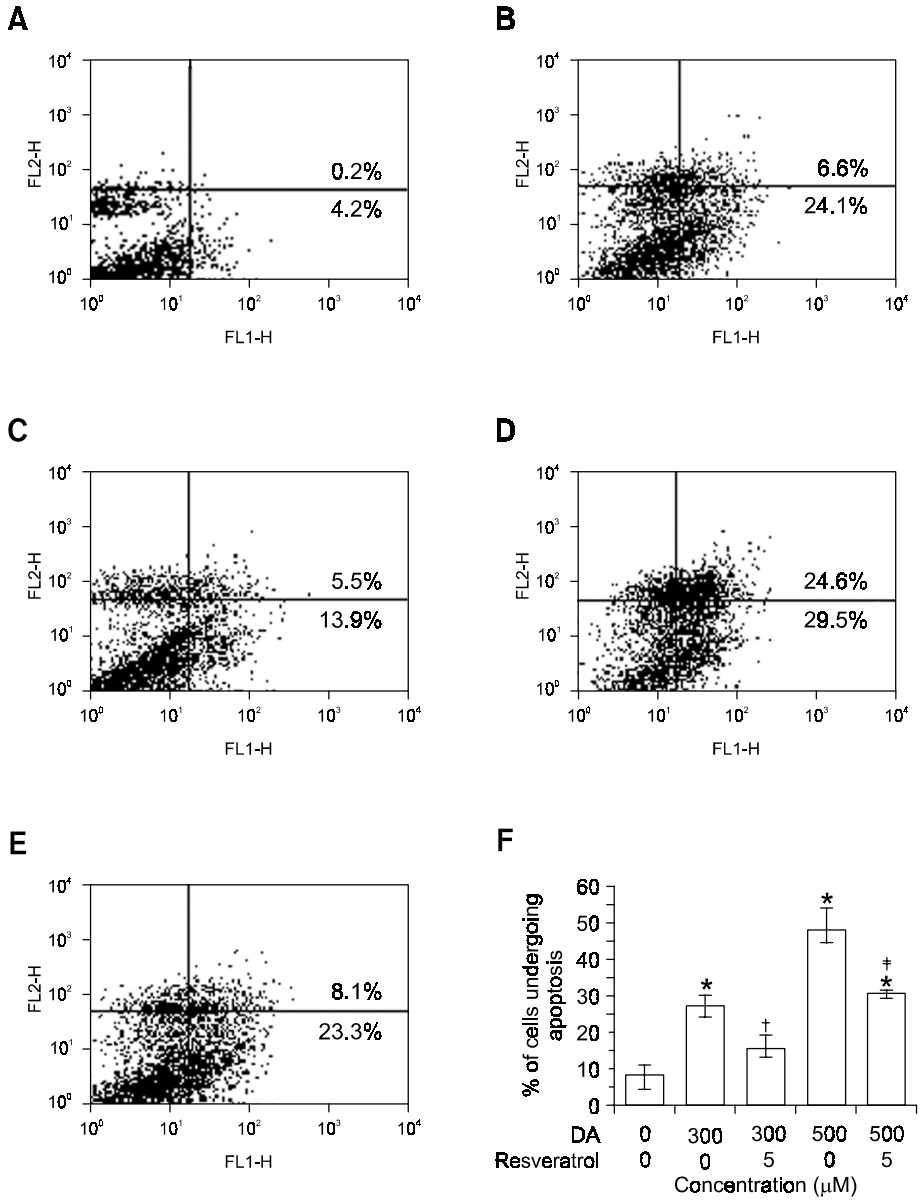

Figure 4. Effect of resveratrol on DA-induced apoptosis in SH-SY5Y cells measured by flow cytometry. Flow cytometric analysis of SH-SY5Y cells stained with Annexin V and PI performed 24 h after cells were exposed to the experimental condition. The number in the lower right quadrant denotes the percentage of early apoptotic cells, and the number in the upper right quadrant denotes the percentage of late apoptotic cells. The results shown in (F) are the standard error of the mean for three independent experiments. (A): no treatment; (B): 300 μM DA; (C): 300 μM DA + 5 μM resveratrol; (D): 500 μM DA; and (E): 500 μM DA + 5 μM resveratrol. For statistical analysis, we conducted a Student paired *t*-test. **P* < 0.01 compared with control cells. †*P* < 0.001, ‡*P* < 0.01 vs. DA 300 μM or 500 μM treated cells.

Discussion

Previous studies indicate that DA, although it is an essential neurotransmitter under physiological conditions, may participate in neurodegenerative processes as a neurotoxin (Luo *et al*., 1998). Oxidative stress has been proposed as a major source of dopaminergic neuronal degeneration in PD, and one of the possible causes for oxidative stress is ROS generated during normal DA metabolism (Junn and Mouradian, 2001). The oxidative products of DA may function as stimuli to activate caspases and subsequent apoptosis, similar to other environmental stresses including UV radiation, ionizing radiation and heat shock (Verheij *et al*., 1996). In contrast, polyphenols possesses free radical scavenging properties and thus, they may have cytoprotective actions in several organs such as kidney and brain (Skrzydlewska *et al*., 2005; Jang *et al*., 2006). Resveratrol is a known polyphenolic compound naturally present in grapes, wine, and peanuts (Ulrich *et al*., 2005), and it has shown to exert antioxidant, anti-inflammatory, antimutagenic, and anticarcinogenic effects (Jang *et al*., 1997; Soleas *et al*., 1997; Surh, 1999).

Our data showed that DA significantly induces the cell death in time and dose dependent manner (Figure 1A and B) and DA-induced cytotoxicity was

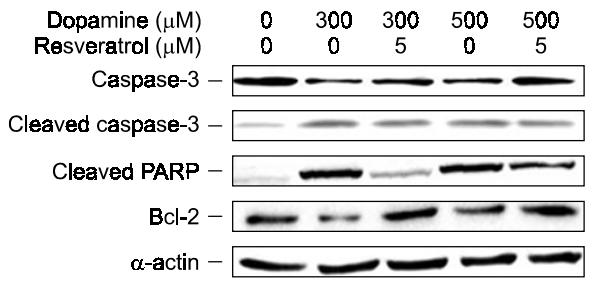

Figure 5. Effect of resveratrol on the DA-induced apoptotic signaling. SH-SY5Y cells were incubated with 300 μM or 500 μM DA for 24 h in the presence or absence of 5 μM resveratrol and harvested for Immunoblot analysis. The protein of Bcl-2, caspase-3, cleaved form of caspase-3 and PARP were determined using immunoblot analysis with corresponding antibodies. Actin was used as an internal control. Each blot is representative of three independent experiments.

partially restored by 3-5 μM resveratrol (Figure 1C). The cytoprotective effects of resveratol appeared to be better by low concentration resveratrol than by high concentration resveratrol of 10-25 μM (data not shown). In previous study, resveratrol was actually shown to have dual functions at different concentrations: High concentrations of resveratrol (40-160 μM) induce apoptosis, whereas cell proliferation increased at low concentrations of resveratrol (up to 10 μM) (Stervbo *et al*., 2006).

In the present study, DA caused apoptotic cell death with significant morphological nuclear changes (Figure 2), consistent with previous *in vitro* studies (Emdadul Haque *et al*., 2003). To examine the role of DA for SH-SY5Y cell apoptosis, change of MMP was traced in the experimental conditions. Maintenance of MMP is necessary for production of energy (ATP) and preservation of cellular homeostasis, and is a critical primary determinant of cell survival (Akao *et al*., 2003). We confirm that DA causes dissipation of MMP and that inhibition of MTS conversion and disruptions in mitochondrial functions may be involved in the mechanisms of DA-induced apoptosis in SH-SY5Y cells. We now show that this effect is inhibited by pretreatment with resveratrol (Figure 3), and that the protective effect of resveratrol against DA-induced apoptosis might be related to the production of ATP and the preservation of cellular homeostasis.

A key event during the early stage of apoptosis is that PS of the inner leaflet of the cell membrane appears on the outer leaflet. Annexin V preferentially binds to PS and can be used to detect expression of PS on the surface of apoptotic cells. PI diffuses into necrotic cells (Nicolini *et al*., 2001), and was used in these experiments as an indicator of lost of cell membrane integrity. Membrane permeability by dual staining with Annexin V and PI revealed that high concentrations of DA (300 and 500 μM) significantly damaged plasma membrane integrity. These deleterious effects on SH-SY5Y cells can be reversed by resveratrol (Figure 4).

The decrease in MMP is a necessary step for inducing cytochrome c release and caspase-3 activation in many apoptotic events (Perkins *et al*., 2000; Qiu *et al*., 2000). Caspases play an important role in the apoptotic process by two distinct pathways: the death receptor pathway and the mitochondrial pathway (Markus, 2000). Whichever pathway is involved, caspase-3 acts as an apoptotic executor. Caspase-3 activates DNA fragmentation factor, which in turn activated endonucleases to cleave nuclear DNA, and ultimately leads to cell death (Wang and Xu, 2005). In this study, treatment with DA led to an increase in caspase-3 activity, but resveratrol pretreatment effectively suppressed the caspase-3 activation. Therefore, resveratrol may act upstream of caspase-3 to block apoptosis.

By virtue of measuring the proteolytic cleavage of nuclear enzyme PARP that is the down-stream target of caspase-3, apoptosis signaling event between DA and resveratrol was tested. Proteolytic cleavage of PARP has been shown to occur during 2,2',5,5'-tetrachlorobiphenyl-induced apoptosis in human neuronal SK-N-MC cells (Hwang *et al*., 2001), and staurosporine-induced apoptosis in dopaminergic neurons (Kim *et al*., 1999). On activating caspase-3, DA treatment induced the cleavage of PARP. The activation of caspase-3 as well as the cleavage of PARP that follow DA treatment was also prevented completely by the co-incubation with resveratrol (Figure 5).

Bcl-2 is a key member of the anti-apoptotic Bcl-2 family that plays a key role in regulating mitochondrial-mediated apoptotic cell death (Marchetti *et al*., 1996; Yang *et al*., 1997; Tsujimoto, 1998). Bcl-2 has been shown to attenuate caspase-3 activation (Sawada *et al*., 2000; Yamakawa *et al*., 2000), and overexpression of Bcl-2 can protect neuronal cells from neurotoxic insult (Borg and London, 2002; Howard *et al*., 2002; Saito *et al*., 2003). In this study, the Bcl-2 protein decreased in the SH-SY5Y cells treated with DA, whereas it increased in cells pretreated with resveratrol. These results suggest that DA induces apoptosis through canonical apoptosis signal, and it is possible for resveratrol to suppress apoptotic cell death signals induced by DA.

In summary, DA caused apoptosis in SH-SY5Y cells through the induction of oxidative stress pathways, and this apoptotic effect can be ameliorated by resveratrol pretreatment in a specific, low dose range. The elucidation of intracellular signal in response to DA-induced oxidative stress and their mo-

dulation by resveratrol may provide additional insights into the molecular basis of the neuroprotective effects of this antioxidant phytochemical. Whether DA contributes significantly to the development of PD, needs further studies as does whether resveratol or related polyphenolic compounds may have a role in prevention of the advancement of PD, remains to be determined.

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