Potential redox-sensitive Akt activation by dopamine activates Bad and promotes cell death in melanocytes

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Dopamine (DA) is a well known oxidative neurotoxin. In addition, Akt has been reported to deliver a survival signal that inhibits apoptosis. However, it has also been reported that chronic Akt activation leads to apoptosis in response to oxidative stress. The objective of the present study was to investigate the possible role of the Akt pathway in vitiligo and its possible relationship with DA-induced cell death using Mel-Ab cells. Cultured Mel-Ab cells were treated with DA with and without N-Acetyl-L-cysteine (NAC), which is known to have antioxidative properties. Cell viability was then assessed by a crystal violet assay and Annexin staining was performed. The changes in the expression of Akt were analyzed by western blot analysis. The cell viability was reduced by approximately 60% in response to treatment with 500 μM DA, and NAC effectively prevented this cytotoxic effect. Likewise, treatment with DA produced numerous Annexin positive cells, while treatment with NAC prevented this apoptotic cell death. Akt was slowly phosphorylated after treatment with DA, while NAC clearly inhibited the DA-induced Akt activation. Western blot analysis also showed that treatment with DA induced the activation of Bad. Finally, LY294002 exerted a protective effect against DA-induced apoptotic cell death. DA may induce redox-sensitive Akt activation and increase the level of Bad, which can promote cell death by heterodimerization with survival proteins. Moreover, NAC effectively protects against DA-induced melanocyte death via inhibition of DA-induced Akt activation.

Introduction

Oxidative stress has been implicated in the pathophysiology of multiple human diseases.[1] Furthermore, oxidative stress is regarded as a mediator of nerve cell death in several neurodegenerative disorders.[2]

Vitiligo is characterized by the selective destruction of melanocytes which had been derived from neuroectoderm. Although its cause is unknown, several reasons for the selective destruction of melanocytes have been suggested. Dopamine (DA) is a well-known neurotoxin that plays an etiologic role in neurodegenerative disorders such as Parkinson's disease, and it has been reported that DA induces oxidative stress and neuronal cell death.[3,4] Neural factors have long been suspected to contribute to the development of vitiligo, and catecholamines such as norepinephrine, epinephrine, and DA and their metabolites have been found to be elevated in the urine and plasma of vitiligo patients.[5-7] We previously reported that melanocytes were susceptible to DA, and that thiol compounds such as N-acetyl-L-cysteine (NAC) effectively protected against DA-induced melanocyte cell death.[8] Using this model, the role of Akt in DA-induced cell death was investigated.

Activation of Akt is known to deliver a survival signal that inhibits apoptosis.[9] It has also been reported that Akt inhibits apoptosis through a variety of molecular mechanisms, including direct phosphorylation and inhibition of the pro-apoptotic Bcl-2 family and Bad.[10] However, chronic Akt activation has also been reported to lead to apoptosis through oxidative stress.[11] In this study, the mouse-derived spontaneously immortalized melanocyte cell line, Mel-Ab, was used to investigate the possible role that Akt pathway in vitiligo.[12]
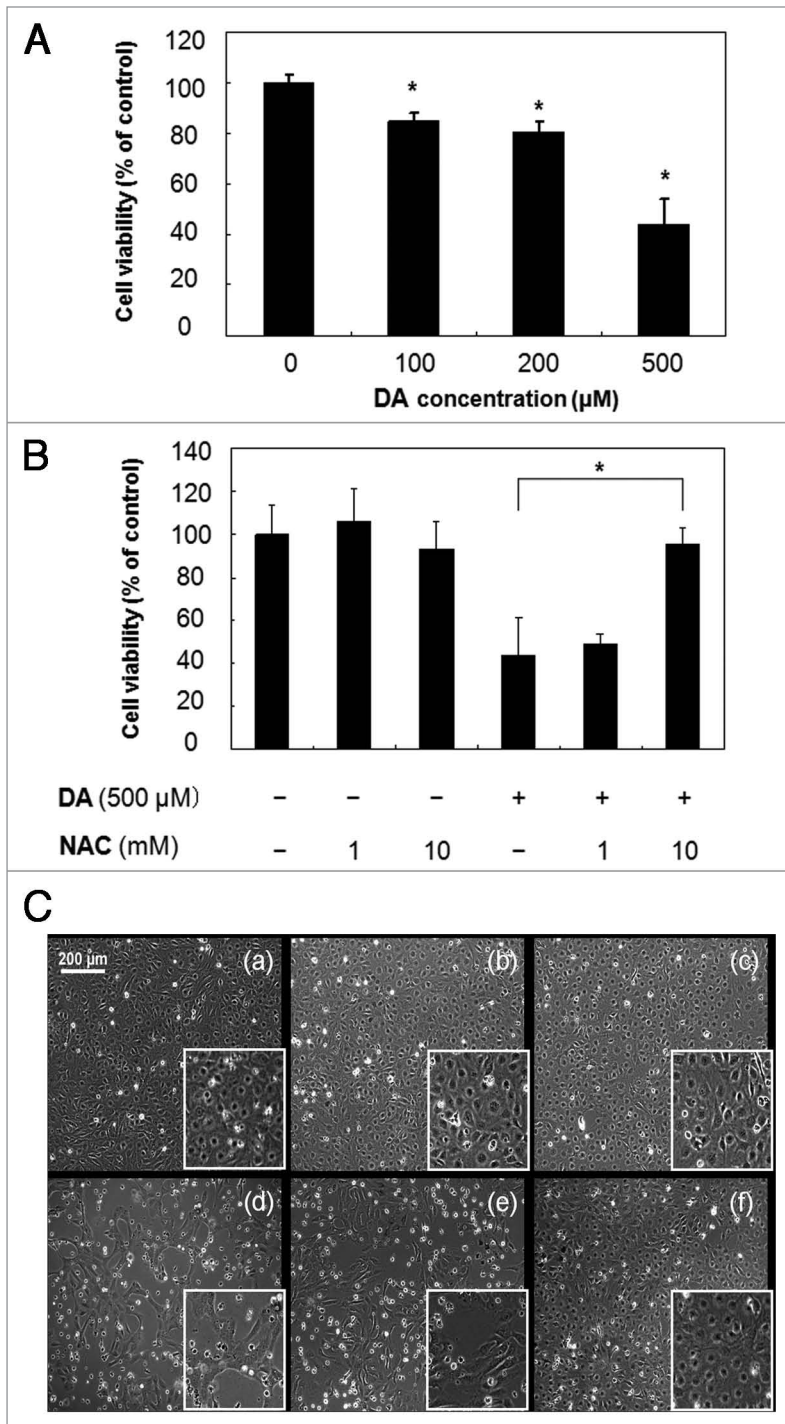
Results

Dopamine-induced cytotoxicity and the effects of NAC. Mel-Ab cells were treated with various concentrations of DA (0–500 μM) for 24 hr, after which the cell viability was measured. The cell viability was reduced by approximately 60% in response to treatment with 500 μM DA; therefore, this concentration was chosen for further experiments (**Fig. 1A**). It was previously reported that NAC and glutathione protected Mel-Ab cells against dopamine-induced cell death.[8] Thus, the protective effects of NAC were evaluated. The results revealed that NAC effectively

Figure 1. Concentration-dependent cytotoxic effects of DA and protective effects of NAC against DA-induced cytotoxicity . (A) Following serum starvation, the cells were treated with various concentrations of DA for 24 hr, after which their viability was measured by crystal violet assay. (B) After serum starvation, the cells were pre-treated with different concentrations of NAC for 20 min and then treated with 500 μM DA for 24 hr. Next, the viability of the cells was measured by crystal violet assay. (C) The morphology of the cells was evaluated using an inverted microscope (x100 objective). (a) control; (b) 1 mM of NAC; (c) 10 mM of NAC; (d) 500 μM of DA; (e) 1 mM of NAC and 500 μM of DA; (f) 10 mM of NAC and 500 μM of DA. *p < 0.05 (Mann-Whitney U test).

prevented the DA-induced cytotoxicity. The survival rate was found to be greater than 95% in the presence of 10 mM NAC (**Fig. 1B**). These findings were confirmed by microscopic examination (**Fig. 1C**). Thus, 10 mM NAC was used for subsequent experiments.

Dopamine-induced apoptotic cell death and the effects of NAC. Early events in the apoptotic process include the translocation of phosphatidylserine. Thus, Annexin V can bind to phosphatidylserine on the surface of cells undergoing apoptosis.[13] The present study showed that DA treatment produced numerous Annexin-V positive cells, and that NAC effectively prevented the formation of Annexin-V positive cells (**Fig. 2A**). In addition, PARP were clearly activated after DA treatment, and NAC prevented this activation of PARP (**Fig. 2B, C**).

Akt activation after dopamine treatment and the effects of NAC. To investigate the mechanism underlying DA-induced cell death, the changes in Akt were analyzed by western-blotting. As shown in **Figure 3A**, immediate changes were not observed; however, Akt phosphorylation was slowly increased by time-dependent manner after DA treatment. We also evaluated the effects of NAC to determine if it could inhibit the DA-induced Akt activation. As shown in **Figure 3B**, NAC clearly inhibited DA-induced Akt activation.

Protective effects of the Akt pathway inhibitor. The role of Akt pathway in the induction of DA-induced cell death was evaluated. Cell viability assay (**Fig. 4A**) showed that LY294002 partially protected against DA-induced apoptotic cell death. In addition, cell death was evaluated by flow cytometric analysis. After DA treatment, cells were analyzed by forward scattering and side scattering. Results showed that DA treatment induced the appearance of cells (R1) at the right side of normal cells. Thus, these cells are considered to be apoptotic cells. Then, LY294002 was added to test the effects of inhibition of Akt pathway in DA-induced cell death. It was found that probable apoptotic cell death has decreased from 33.5% to 15.0% (**Fig 4B, C**).

Involvement of Akt and Bad in the induction of DA-induced apoptotic cell death. Western blotting demonstrated that the DA induced activation of Bad, while NAC almost completely abolished the DA induced activation of Bad. LY294002 also partially inhibited the DA induced activation of Bad (**Fig. 5A**). However, Bcl-2 and Bcl-xL were not changed (**Fig. 5B, C**).

Discussion

Oxidative stress is believed to be a key mediator of neurotoxicity by DA. The auto-oxidation of DA produces quinines as well as an array of other free
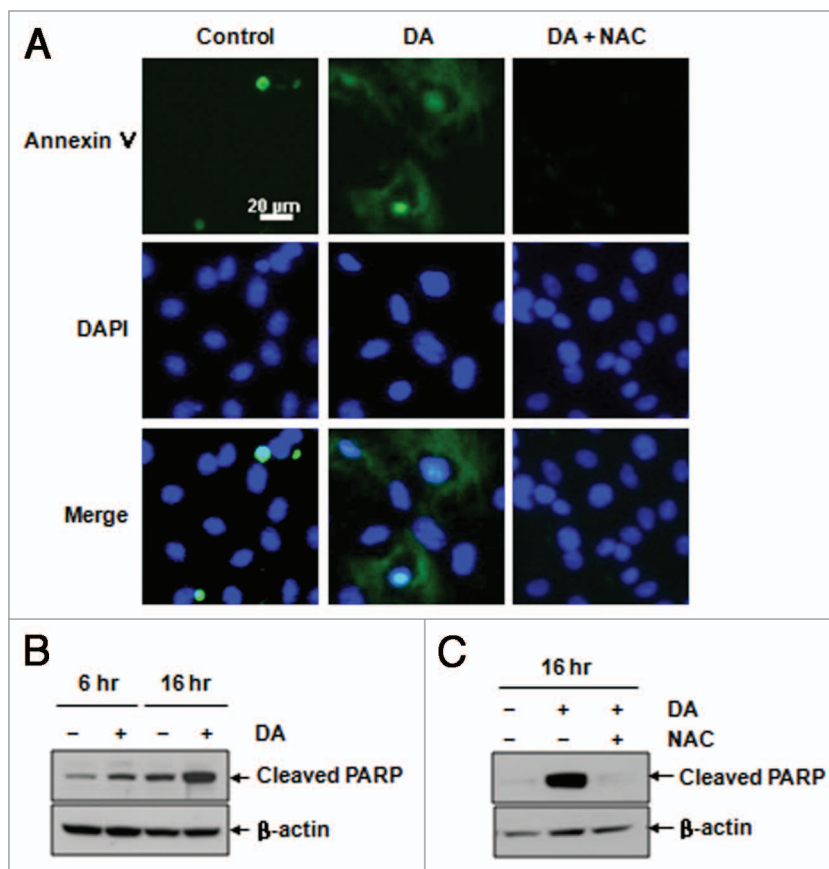
radical species. Additionally, monoamine oxidase (MAO) contributes to DA toxicity via the generation of hydrogen peroxide and 3,4-dihydroxyphenylacetaldehyde (DOPAL). These ROS and quinines induce cellular damage and apoptosis.[14] Previously, we reported that DA induced apoptotic cell death in Mel-Ab cells and cultured normal human melanocytes. Furthermore, we showed that thiol-containing compounds, such as NAC and GSH, induced a marked protective effect against apoptosis, while vitamin C, vitamin E, Trolox and quercetin had negligible protective effects.[8]

In the present study, the molecular mechanism of cell death following DA treatment was investigated. The results showed that delayed activation of Akt occurred following DA treatment (**Fig. 3**). It has previously been reported that phosphotidylinositol 3-kinase regulates melanogenesis by modulating the expression of tyrosinase, and that activation of Akt is related to the suppression of melanin production in G361 melanoma cells.[15]
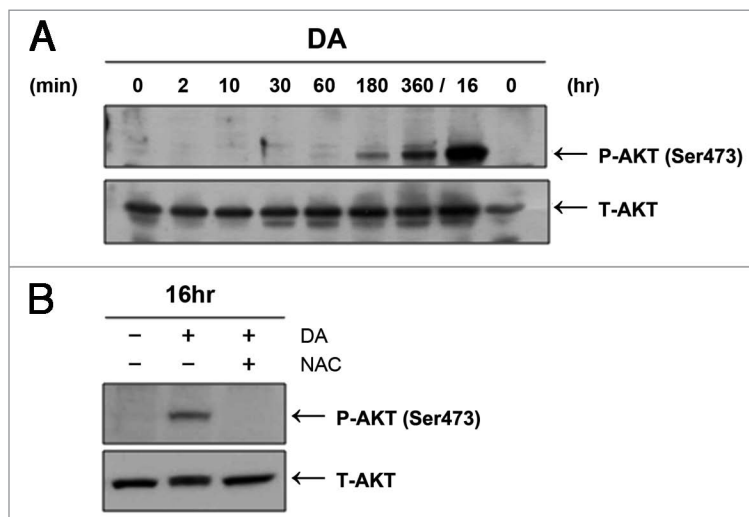
It has also been reported that Akt is a major mediator of the cell survival pathway; therefore, activation of Akt leads to the inhibition of caspase activity and protection from apoptotic cell death.[16] Despite this, stimulation of the Fas receptor by its ligand (FasL) induces phosphorylation of Akt and a parallel increase in apoptosis in epidermal CI41 cells.[17] Furthermore, electron resonance studies have shown that FasL treatment induces rapid generation of reactive oxygen species (ROS), and that inhibition of ROS by antioxidants effectively inhibits Akt signaling. As already indicated, increased Akt is a hallmark of antiapoptotic survival signals.[18] However, oxidative stress related Akt activation leads to apoptotic cell death.[11] Taken together, these findings suggest that DA also leads to redox sensitive Akt activation and apoptotic cell death.
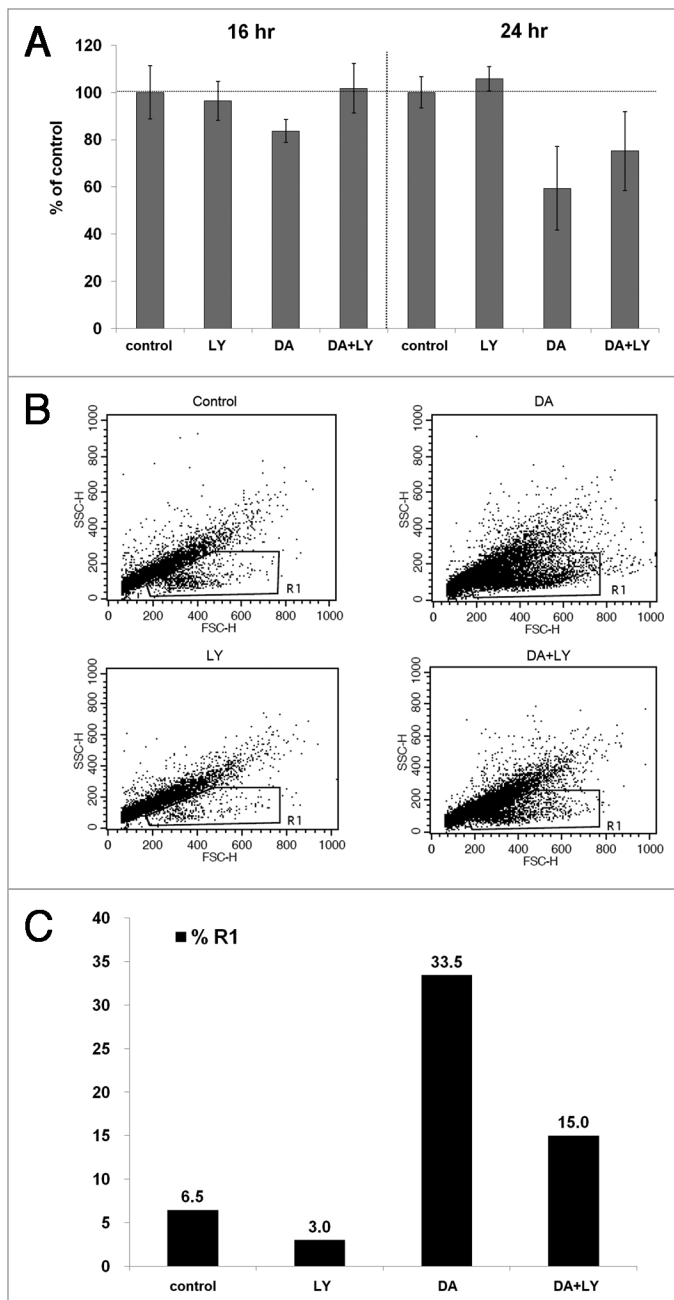
In mammals, three Akt isoforms Akt1, Akt2, Akt3 which exhibit sequence homology are known. Akt1 is the most ubiquitously expressed isoform and Akt2 has its highest expression levels in insulin-responsive tissue.[19,20] Akt3 expression is mainly limited to brain, testis, lung, heart, kidney, mammary gland and fat.[21] Since, several previous studies showed that Akt1 is involved in apoptosis induced by oxidative stress11 or in the redox

Figure 2. Microscopic observation of DA-induced cell death and PARP activation by western blotting. (A) After serum starvation, the cells were treated with 500 μM DA for 16 hr with or without pretreatment with 10 mM NAC for 20 min. Photographs were then taken using a fluorescent inverted microscope (x100 objective). (B) After serum starvation, the cells were treated with 500 μM DA for 6 hr or 16 hr. β-actin antibody was used as a control for equal loading of proteins. (C) The cells were then treated with 500 μM DA for 16 hr with or without 10 mM NAC pretreatment. β-actin antibody was used as a control for equal loading of proteins.

Figure 3. Activation of Akt after DA treatment. (A) After serum starvation, the cells were treated with 500 μM DA for the indicated time points. Total-Akt antibody was used as a control for equal loading of proteins. (B) After serum starvation, the cells were treated with 500 μM DA for 16 hr with or without pretreatment with 10 mM for 20 min. Total-Akt antibody was used as a control for equal loading of proteins.

Figure 5. Levels of Bad, Bcl-2 and Bcl-xL in DA-induced apoptosis. (A) After serum starvation, the cells were treated with 500 μM DA for 16 hr with or without pretreatment with 10 mM NAC or 50 μM LY294002. β-actin antibody was used as a control for equal loading of proteins. (B, C) Western blotting was conducted using Bcl-2 (B) or Bcl-xL (C) antibody. β-actin antibody was used as a control for the equal loading of proteins.
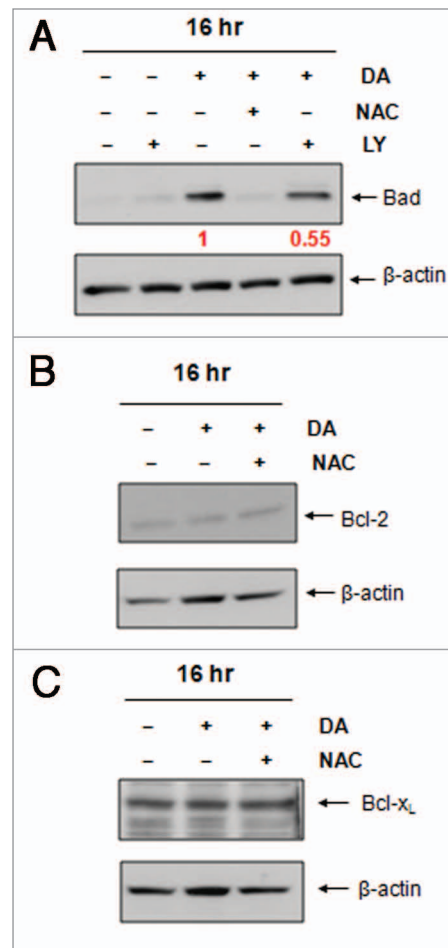
Figure 4. Effect of Akt inhibitor on DA-induced cell death. (A) After serum starvation, the cells were treated with 500 μM DA for 16 hr or 24 hr with or without pretreatment with 50 μM LY294002 for 20 min. Viability of the cells was measured by crystal violet assay. *p < 0.05 (Mann-Whitney U test). (B, C) After serum starvation, the cells were treated with 500 μM DA for 16 hr with or without pretreatment with 50 μM LY294002 for 20 min. Flow cytometric analyses were measured by forward and side scattering. Graph shows the percentages of representative data among two independent experiments.

modulation of cell cycle progression,[22] we could presume that the Akt which detected in our study might be Akt1.

To determine whether DA induced apoptotic cell death via the Akt pathway, Mel-Ab cells were treated with DA with or without LY294002 (a specific inhibitor of the Akt pathway)

pretreatment for 20 min. The results revealed that LY294002 partially inhibited the DA-induced cell death (**Fig 4, 5**). These findings indicate that the Akt pathway is involved in DA-induced cell death. Furthermore, NAC, which effectively protects against DA-induced cell death (**Fig. 1**), also inhibits DA-induced Akt activation (**Fig. 3**). Taken together, these findings indicate that DA may induce apoptotic cell death through the activation of Akt, which is considered to be redox sensitive.

It has also been reported that proteins belonging to the Bcl-2 family are important regulators of cell death in mammalian cells.[23] Bad is a distinct member of the Bcl-2 family that promotes cell death, at least in part, through heterodimerization with the survival proteins, Bcl-2 and Bcl-xL.[24] The proapoptotic function of Bad is reported to be regulated by the Akt pathway.[10] Recently, it is also reported that Myricetin, a potent anticancer phytochemical, caused a decrease in phosphorylation of Bad by decreasing phosphorylation of Akt.[25] Furthermore, it is reported that transforming growth factor-beta prevent osteoblast apoptosis

via PI3K/Akt and p-Bad signaling. These report showed that cell survival signaling is closely related to PI3K/Akt and p-Bad signaling.[26] The results of the present study showed that DA increased the levels of Bad, but it had no effect on the levels of Bcl-2 and Bcl-xL (**Fig. 6**). These results suggest that DA-induced Bad promotes cell death through heterodimerization with Bcl-2 and Bcl-xL.

In conclusion, DA can induce redox-sensitive Akt1 phosphorylation and upregulation of Bad, which may promote melanocyte cell death in vitiligo.

Materials and Methods

Reagents. Dopamine hydrochloride (DA, H8502) and N-Acetyl-L-cysteine (NAC, A8199) were purchased from Sigma-Aldrich, Inc. (St. Louis, MO). Antibodies recognizing cleaved PARP (#9544), Bcl-2 (#2876), Bad (#9292), and β-actin (#4967) were obtained from Cell Signaling Technology, Inc. (Danvers, MA). Antibodies recognizing phospho-Akt1/2/3 (Ser473, sc-7985-R), total-Akt (sc-8312) and Bcl-x$_L$ (sc-7195) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). LY294002 (PHZ1144) was purchased from Invitrogen (Carlsbad, CA).

Cell cultures. Mel-Ab cells, a mouse-derived spontaneously immortalized melanocyte cell line that produces large amounts of melanin,[12] were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS, SH30070.03, Hyclone, Thermo Fisher Scientific Inc., Waltham, MA), 100 nM 12-O-tetradecanoylphorbol-13-acetate (TPA, P8139, Sigma), 1 nM cholera toxin (C8052, Sigma), and 1x antibiotic-antimycotic (#15240-062, Invitrogen) at 37° under 5% CO$_2$.

Crystal violet assay. Cell viability was determined by a crystal violet assay.[12] After removal of the culture media, cells were stained with 0.1% crystal violet in 10% ethanol for 5 min at room temperature. The cells were then rinsed five times with distilled water. Next, the crystal violet remaining in the cells was extracted by treatment with 95% ethanol for 15 min at room temperature. The absorbance was then determined at 590 nm using an ELISA reader (Spectra max plus 384, Molecular Devices, Sunnyvale, CA).

Protective effects of antioxidants. To investigate the cytoprotective activity of NAC, Mel-Ab cells were seeded onto 6-well plates (1 x 105 cells/well) or 24-well plates (2 x 104 cells/well). After serum starvation (DMEM-0.1% BSA) for 24 hr, the cells were pretreated with NAC (1 or 10 mM) for 20 min. Freshly prepared DA solution (500 μM) was then added to each well, after which the samples were incubated for 24 hr.

Morphological examination. The morphology of the cultured Mel-Ab cells was observed using a fluorescent inverted microscope (Axio Observer. Z1, Carl Zeiss, Thornwood, NY). Morphological changes were evaluated at 100x magnification using the Axio Vision 4.7 software (Carl Zeiss).

Annexin staining. After treatment, the cells were washed using HEPES buffer and then incubated with 2% Annexin-V-Fluorescein (#11 858 777 001, Roche, Mannheim, Germany) in HEPES buffer for 15 min at room temperature. The stained cells were then rinsed with methanol and incubated with 1 μg/ml DAPI (#10 236 276 001, Roche) to counter-stain the nucleus. After washing, the cells were observed using a fluorescent inverted microscope.

Western blot analysis. Cells were lysed in buffer composed of 62.5 mM Tris–HCl (pH 6.8), 2% SDS, 5% β-mercaptoethanol, 2 mM phenylmethylsulfonyl fluoride, 1 mM Na3VO4, 50 mM NaF and protease inhibitors (Roche). The total protein concentration was determined using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA), after which 50 μg of proteins were electrophoresed using Tris-Glycine gel (EC6008, Invitrogen). The separated proteins were then transferred to a PVDF membrane (Immobilon-P, IPVH00010, Millipore, Bedford, MA). The membrane was then blocked using 5% non-fat dry milk (Bio-Rad) in Tris-buffered Saline containing 0.2% Tween 20. Next, the blocked membrane was incubated with primary antibody and horseradish peroxidase-conjugated secondary antibody. The bound antibodies were detected using an ECL western blotting analysis system (RPN2109, Amersham International, Little Chalfont, UK).

Flow cytometric analysis. Sixteen hours after DA treatment, we collected the culture supernatant, which contained floating dying cells and apoptotic cells. Then adherent cells were harvested by a brief trypsinization. The two fractions were combined and washed with PBS. Flow cytometric analyses were performed on a FACSCalibur (Becton Dickinson, San Jose, CA). Experiments were repeated at least twice under the same conditions and settings. The effects of LY294002 (50 μM) pretreatment were also investigated.

Statistics. Differences between groups were analyzed by a Mann-Whitney U test. The SPSS statistics package (SPSS V12.0K, SPSS Inc., Chicago, IL) was used for all analyses.

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