4,5-dianilinophtalimide protects neuroendocrine cells against serum deprivation-induced stress and apoptosis

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Abstract

OBJECTIVE: The aim of this study was to reveal the effects of 4,5-dianilinophthalimide (DAPH), which inhibits amyloid β fibrillization, against serum deprivation (SD)-induced apoptosis and the possible mechanisms in differentiated PC12 neuron cells.

METHODS: Firstly, we evaluated whether DAPH protects cell viability exposed to SD by MTT assay. Next, we examined the changes of phospho-p38 MAPK (Thr180/Tyr182), phospho-HSP27 (Ser82), phospho-c-JUN (Ser73) and cleaved-CASP3 (Asp175) profiles by immunoblotting, in PC12 cells exposed to SD. Intracellular reactive oxygen species (ROS) level was also measured.

RESULTS: SD induced apoptosis accompanied by up-regulation of phospho-p38 MAPK (Thr180/Tyr182), phospho-HSP27 (Ser82), phospho-c-JUN (Ser73), cleaved-CASP3 (Asp175) and intracellular ROS content. Co-treatment with non-toxic doses of DAPH prevented apoptosis by the attenuation of activated proteins and reduction of ROS level. These results suggest that serum deprivation-induced apoptosis inhibited by DAPH administration.

CONCLUSION: We have provided for the first evidence that DAPH has a neuroprotective effect on SD-caused stress, probably via contributing the re-establishment of redox homeostasis.

Abbrevations:

BCA	- Bicinchoninic acid
c-JUN	- Proto-oncogene c-JUN
CASP3	- Caspase-3
DAPH	- 4,5-Dianilinophthalimide
DCF	- 2',7'-dichlorofluorescein
DCF-DA	- 2',7'-dichlorofluorescein diacetate
GAPDH	- Glyceraldehyde-3-phosphate dehydrogenase
HSP27	- Heat shock protein 27
JNK	- c-jun N-terminal kinase
IC50	- Half maximal inhibitory concentration
MAPKAPK2	- MAP kinase-activated protein kinase 2
MTT	- Thiazoyl blue tetrazolium bromide
NGF	- Nerve growth factor
p38 MAPK	- p38 mitogen activated protein kinase
PMSF	- Phenylmethanesulfonyl fluoride
PVDF	- Polyvinylidene difluoride
ROS	- Reactive oxygen species
SD	- Serum deprivation

ORIGINAL ARTICLE

INTRODUCTION

Serum is essential for most of the cultured cell lines. Serum constituents such as growth factors, minerals, lipids and numerous other factors are crucial for cell growth and differentiation (van der Valk *et al.* 2004). In addition to this, it is well known that the deprivation of serum from medium induces apoptosis in cultured cells. Besides being one of the factors of ischemia (Bialik *et al.* 1999), removal of serum from culture medium causes massive neuronal cell death (Greene 1978); on the other hand, different studies demonstrated that antioxidant treatment has an inhibitory effect on serum deprivation (SD)-induced apoptosis (Ferrari *et al.* 1995). While the exact mechanism is still unknown, there have been significant amount of reports indicating that SD-induced apoptosis is correlated with increased ROS, such as superoxide and hydrogen peroxide (Satoh *et al.* 1996; King *et al.* 2003; Pandey *et al.* 2003; Zhuge & Cederbaum 2006). In fact, the SD-triggered intracellular signalling pathways are not yet completely clear, however, various stress-related enzymes, such as caspases and HSPs have apoptotic or protective roles (Stetler *et al.* 2010; Higuchi *et al.* 2006).

Neuronal cell death is a characteristic of neurodegenerative diseases that occurs mainly by necrosis and/or apoptosis (Lipton 1999; Mattson 2000; Yuan *et al.* 2003) and neuronal cells deprived of serum go through apoptotic cell death (LeBlanc *et al.* 1999; Howard *et al.* 1993). PC12, a neuroendocrine cell line, which differentiates into a neuronal phenotype when exposed to nerve growth factor, is a useful model system for neuronal differentiation, has been extensively used for intracellular signaling studies (Vaudry *et al.* 2002). This cell line allows rapid screening of different molecular pathways with minimal preparation time and comprises a convenient model for studying ischemia, neuronal apoptosis and its prevention (Hillion *et al.* 2005; Lee *et al.* 2012). The cells respond to environmental stresses through various mechanisms ranging from initiation of prosurvival strategies to activation of apoptotic pathways. In this context, there are many stress/apoptosis marker proteins, which are involved in normal signaling and survival pathways, and these marker proteins also interact with eventual cell death pathways. In this present study we assessed the effects of 4,5-dianilinophthalimide (DAPH), a selective inhibitor of formation of Aβ42 fibers and prions associated with various neurodegenerative diseases such as Alzheimer's disease (Blanchard *et al.* 2004; Wang *et al.* 2008), on the expressional profiles of several stress/apoptosis marker proteins in serum deprived PC12 cells. We found that DAPH is significantly effective on decreasing stress-related and apoptotic conditions triggered by serum deprivation.

MATERIAL & METHODS

Cell culture

PC12 cell line, stably overexpressing NGF receptor (PC12 6–15) has been used (Hempstead *et al.* 1992). This cell line has been kindly provided by Dr. V. Laketa (EMBL, Germany). Cells were maintained in vitro using RPMI medium supplemented with 10% heat-inactivated horse serum, 5% FBS, 2 mM L-glutamine and 1% penicillin-streptomycin (complete medium). Cells were cultured in a humidified atmosphere of 5% CO2 and 95% air at 37 °C. This cell line responds reversibly to NGF (Promega, Madison, WI, USA) and differentiate into neuronal phenotype when plated on Collagen type IV (Sigma-Aldrich, Steinheim, Germany) coated culture flasks in RPMI medium supplemented with 1% heat-inactivated horse serum, 2 mM L-glutamine, 1% penicillin-streptomycin and 100 ng/mL NGF (differentiation medium). All assays are performed on 48 h-differentiated cells in serum deprived (SD) medium.

Cell viability assay

Cell viability is ascertained using Thiazoyl blue tetrazolium bromide (MTT; Bioworld; Dublin, OH, USA) assay. Briefly, PC12 cells (1×10^4) seeded on collagen-coated 96-well plates were incubated for 48 h in differentiation medium. Next, SD medium containing different concentrations of DAPH (CGP52411; Tocris Bioscience, Avonmouth, United Kingdom) were added. At the end of incubation, 10 µL of MTT stock solution (5 mg/mL) was added, and the plates were incubated at 37 °C for 4 h. Culture medium was removed, the resultant formazan crystals were dissolved in 100 µL DMSO, and the absorbance values were read on a microplate reader SpectraMax M5e (Molecular Devices, Sunnyvale, CA, USA) at 572 nm wavelength. Cells were assayed in hexaplicate, and three independent experiments were carried out.

Measurement of cellular oxidative stress

The accumulation of intracellular ROS was determined by measuring 2′,7′-dichlorofluorescein (DCF) fluorescence. ROS cause oxidation of 2,7-dichlorofluorescein diacetate (DCFH-DA) to the fluorescent product DCF in the cell. In brief, cells seeded on collagen-coated 12-well plates and differentiated for 48 h, (1×10^5/well) then exposed to DAPH for 24 h at 37 °C in serum-deprived medium without NGF. Cell culture plates were washed twice with PBS and incubated with 10 µM DCFH-DA for 30 min (Molecular Probes, Eugene, OR, USA) in PBS. Then DCFH-DA-containing medium was removed; cells were washed twice and DCF fluorescence was quantified (Ex/Em: 485 nm/535 nm) using a multimode microplate reader SpectraMax M5e (Molecular Devices, Sunnyvale, CA).

Western blotting

For protein expression anaylsis, PC12 cells cultured in 60 mm petri-dishes (Sarstedt, Nürnbrecht, Germany) were lysed in 100 µL of lysis buffer (Cell Signaling Technology, Beverly, MA, USA) supplemented with 1 mM PMSF (Roche Diagnostics, Mannheim, Germany). Protein concentrations were determined using the BCA protein assay (Pierce, Rockford, IL, USA). Protein lysates (20 µg) were heated for 5 min at 94 °C in Laemmli sample buffer containing 5% β-mercaptoethanol and then loaded on 4–15% Tris-glycine SDS-PAGE gels, then transferred electrophoretically onto PVDF membranes. Membranes were blocked with 5% non-fat dry milk for 1 h and incubated overnight at 4 °C with the phospho-HSP27 (Ser82), phospho-c-JUN (Ser73), cleaved CASPASE-3 (Asp175), phospho-p38 MAPK (Thr180/Tyr182) and GAPDH antibodies (Cell Signaling Technology, Beverly, MA, USA). Protein bands were detected with horseradish peroxidase-conjugated anti-rabbit secondary antibodies (Cell Signaling Technology, Beverly, MA, USA) and visualized by West-Femto ECL reagents (Pierce, Rockford, IL, USA). Chemiluminescent signals of immunoblots were documented using Gel Logic 2200 Pro (Carestream Health, Rochester, NY, USA). The net intensity of specific proteins was quantified using Carestream Molecular Image Software.

Statistical analyses

Experiments were performed three times and statistical analysis was conducted using Student's *t*-test. Data are expressed as means ± SD and $p<0.05$ was considered as statistically significant.

RESULTS

Serum withdrawal in the absence and presence of DAPH

Serum starved condition is known to induce cell death in the first 24 h in various cell types, especially in neurons (Li *et al.* 2010). In order to establish the optimal time of SD-induced apoptosis in post-mitotic PC12 cells, a time-dependent study was carried out. We determined that SD induces approx. 40% cell death after 24 h (data not shown). PC12 cells were treated with 0–50 µM DAPH, and cell viability was quantified at 24^{th} h using MTT assay. The results in Figure 1a showed that DAPH reduced cell viability in a dose-dependent manner. The IC_{50} value of 20 µM was obtained at 24 h. To test neuroprotective effect of DAPH on SD-induced cell death, further experiments were performed using non-toxic doses of this chemical lower than value of IC_{50}. Next, we evaluated the neuroprotective effect of DAPH on SD-induced apoptosis in neuronal culture. MTT results confirmed that DAPH has a neuroprotective effect on SD-induced PC12 cells (Figure 1b).

DAPH decreases ROS production in serum deprived cells

A cell membrane-permeable fluorescent dye DCF-DA that is sensitive to oxidation, was used to assess the levels of intracellular oxidative stress after exposure to both SD and DAPH for 24 h. Differentiated and serum starved PC12 cells treated with 10 µM DAPH for 24 h displayed a decreased fluorescence, about 50% when compared with SD group cells ($p<0.05$) (Figure 2).

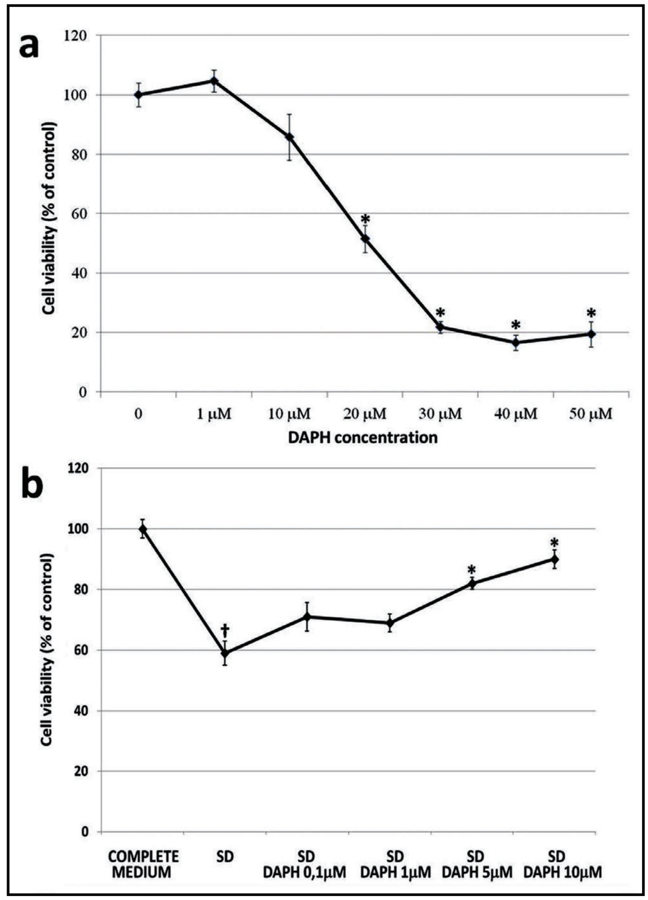

Fig. 1. Cytotoxic levels were assessed by MTT assay. **(a)** Effect of DAPH on non-starved PC12 viability. Differentiated PC12 cells were treated with different concentrations of DAPH (0–50 µM) for 24 hrs, Data are means ± SD. *$p<0.05$ *vs.* 0 (control), n=6, **(b)** Effects of non-toxic concentrations of DAPH on serum-deprived (SD) PC12 viability. Data are means ± SD. †$p<0.05$ *vs.* 0 (control), *$p<0.05$ *vs.* SD, n=6.

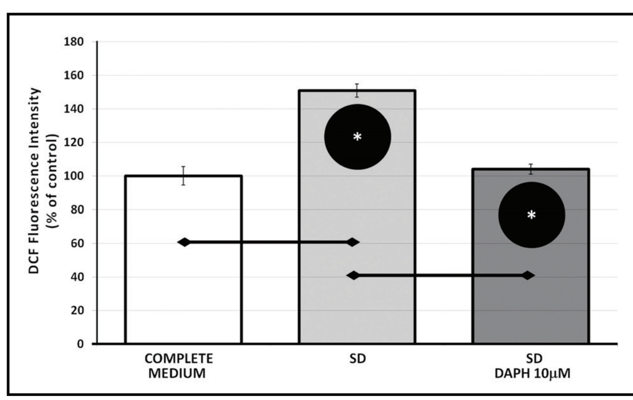

Fig. 2. Effect of SD and DAPH treatment on elevating ROS level of differentiated PC12 cells was determined by spectrophotometrically and showed by DCF fluorescence intensity. Data are means ± SD, *$p<0.05$, (n = 3).

DAPH leads to diminished apoptotis and decreases stress related protein levels

Western blot analysis of PC12 cells exposed to SD revealed expression levels of various proteins, which were markedly downregulated during DAPH treatment (Figure 3a). DAPH co-applied with SD had inhibitory effects on apoptosis compared to SD control. To evaluate the protective effect of DAPH on serum-deprived cells, we first tested the caspase-3 status. Caspase-3 is a critical executioner of apoptosis. Activation of caspase-3 requires proteolytic processing of its inactive zymogen into activated fragments (Nicholson *et al.* 1995). SD itself induced cleavage of pro-caspase-3, resulting in the formation of the 19-kDa active form of this enzyme. Western blot analysis showed that DAPH at a concentration of 5 μM, significantly attenuated the SD-induced formation of the active caspase-3 ($p<0.05$), (Figure 3b). We next examined if p38 MAPK pathway is involved in DAPH protection. p38 is activated by a variety of cellular stresses including inflammatory cytokines, LPS and UV (Rouse *et al.* 1994; Lee *et al.* 1994). In PC12 cells, to evaluate the expression profile of p38

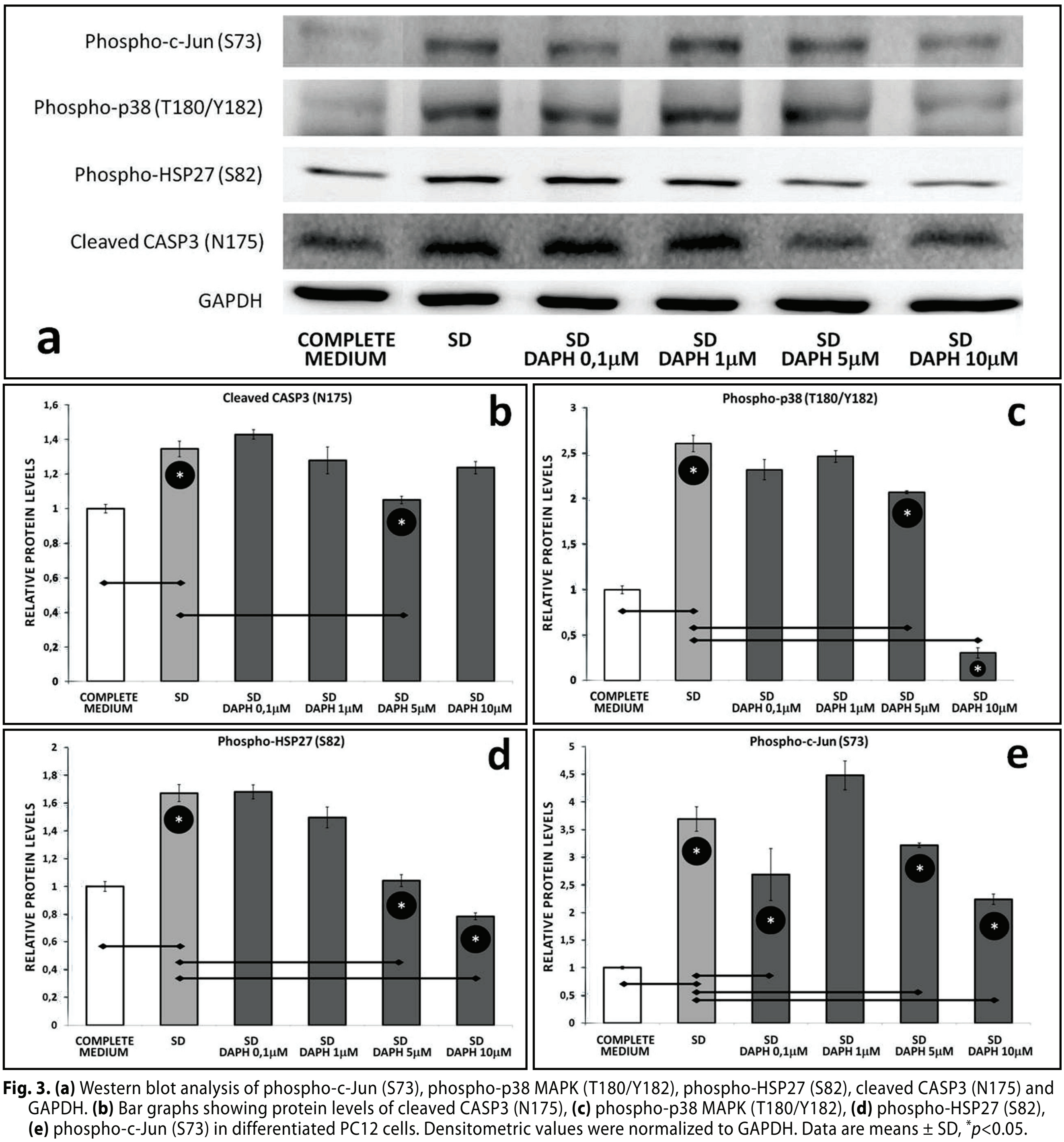

Fig. 3. (a) Western blot analysis of phospho-c-Jun (S73), phospho-p38 MAPK (T180/Y182), phospho-HSP27 (S82), cleaved CASP3 (N175) and GAPDH. **(b)** Bar graphs showing protein levels of cleaved CASP3 (N175), **(c)** phospho-p38 MAPK (T180/Y182), **(d)** phospho-HSP27 (S82), **(e)** phospho-c-Jun (S73) in differentiated PC12 cells. Densitometric values were normalized to GAPDH. Data are means ± SD, $^{*}p<0.05$.

MAPK in SD and DAPH treated conditions, p38 activity was monitored by anti-phospho-p38 (T180/Y182) antibody that specifically binds activated p38. Treatment with DAPH at 5 and 10 μM, significantly reduced p38 phosphorylation, the indicator of p38 activation ($p<0.05$), (Figure 3c). In response to stress, HSP27 expression increases several-fold to create cellular resistance to the adverse environmental stimuli. HSP27 is phosphorylated at Ser15, Ser78, and Ser82 by MAPKAPK-2 as a result of the activation of the p38 MAPK pathway (Rouse *et al.* 1994; Landry *et al.* 1992). Serum deprivation for 24 h caused an increase in activation of HSP27 (Ser82), which was significantly attenuated by addition of DAPH in a dose-dependent manner ($p<0.05$), (Figure 3d). In addition to this, in vitro experiments on neurons have demonstrated that activation of c-JUN by Ser63 and Ser73 phosphorylation can promote apoptosis following serum withdrawal (Ham *et al.* 2000). Hence, we wanted to test whether suppression of c-JUN by DAPH contributed to the neuroprotective effect of DAPH against serum deprivation, and we demonstrated that DAPH has a significant inhibitory effect on c-JUN phosphorylation at its non-toxic concentrations ($p<0.05$), (Figure 3e). Taken together, these results indicate that SD triggers several stress related and proapoptotic pathways in differentiated PC12 cells, on the other hand, DAPH has a neuroprotective effect during serum starvation.

DISCUSSION

SD-induced PC12 cell death was used as an apoptotic model to investigate the therapeutic potential of DAPH as a neuroprotectant in this study. The major finding of this study is that DAPH has a significant antiapoptotic effect on differentiated PC12 cells exposed to SD. The mechanism of this protective action seems to be mediated through reducing activation of CASP3, p38 MAPK, HSP27 and c-JUN. We further showed that the protective mechanisms includes reduction of oxidative stress.

Here we provide the first evidence for direct neuroprotective effect of DAPH against SD-induced apoptosis. It is known that PC12 cell apoptosis is induced by various stimuli, including SD and neurotrophic factor withdrawal. In cultured PC12 cells, it has been shown that SD rapidly induces apoptosis, and some studies reported that SD leads to oxidative stress, which is a mediator of neuronal apoptosis. However, the mechanism by which serum deprivation causes ROS production is not clear; our results are consistent with previously reported studies that claim oxidative stress is involved in SD-induced cell death in PC12 cells (Rukenstein *et al.* 1991; Atabay *et al.* 1996; Lee *et al.* 2010). Moreover, we demonstrated that DAPH administration reversed the oxidative stress status.

Mitogen-activated protein kinases (MAPK) pathways play an important role in cell death and survival. It has been reported that apoptosis induced by withdrawal of trophic factors is mediated by increased p38 MAPK activity which is activated by inflammatory cytokines, environmental stressors, including UV, heat and hyperosmolarity (Han *et al.* 1994; Kummer *et al.* 1997; Kyriakis & Avruch 1996). p38 MAPK has previously been reported to be activated in oxidant-induced apoptosis in cortical neuron model, considered to be a key factor in cell death (Namgung *et al.* 2000). Also p38 MAPK has been shown to promote neuronal cell death in in vivo experimental models of other neurodegenerative diseases (Legos *et al.* 2001; Segura Terros *et al.* 2006) and it has been shown to be activated in patients with Alzheimer's disease (Zhu *et al.* 2001). Our data indicate that the oxidative stress and activation of p38 MAPK attenuated by DAPH treatment.

Activation of p38 MAPK then leads to cleavage of pro-CASP3, yielding active cleaved-CASP3, one of the key effectors of apoptosis (Khreiss *et al.* 2002). It was reported that, upon serum starvation, PC12 cells exhibit increased activation of CASP3, which is considered as an indicator of cell death. (Kim *et al.* 2000) These results are consistent with ours, in addition to this, we found that DAPH induction resulted in decreased formation of cleaved-CASP3.

Upon stimulation by stress, p38MAPK is phosphorylated, which then phosphorylates MAPKAPK2 to phosphorylate and activate HSP27 (Rouse *et al.* 1994). Phosphorylation of HSP27 is observed in response to various stimuli that have either inhibitory (oxidative stress, serum starvation) or stimulatory (serum, mitogens) effects on cell proliferation. Other than that, phosphorylation of HSP27 is under the control of intracellular levels of ROS (Mehlen & Arrigo 1994). We observed that HSP27 is phosphorylated during serum starvation, on the other hand, DAPH exposure to SD cells decreased the expression level of HSP27 phosphorylation. This indicates that DAPH contributed to PC12 survival via decreasing lethal effects of SD-induced ROS, which eliminates the requirment for HSP27 regulated prosurvival pathways.

Several studies reported that JNK is another protein activated by oxidative stress, which is a consequence of SD-induction (Marques *et al.* 2003) and it is considered as an essential molecule in neurodegeneration (Herdegen *et al.* 2001). Our results show that oxidative stress and c-JUN, an important transcription factor that is activated by JNK, are attenuated by DAPH treatment in differentiated PC12 cells. These results suggested that DAPH may act as an anti-oxidant molecule against SD conditions. Taken all together, in the study reported here, we have documented that DAPH has an inhibitory effect on SD-induced stress and apoptosis.

In conclusion, we have provided for the first evidence that DAPH can attenuate serum withdrawal induced apoptosis in neurons. The down-regulation of activated CASP3, p38 MAPK, HSP27 and c-JUN might be responsible for this protective effect. This indicates that DAPH is at least partially contributes to maintenance of cellular homeostasis during serum starvation. Our findings indicate that DAPH has a neuroprotective effect on SD-caused stress, probably via contributing the re-establishment of redox homeostasis. Hence, taken together with the other studies reporting that DAPH has a preventive effect on development of Alzheimer's disease, our results suggest that DAPH could be a potential therapeutic agent for neurodegenerative disorders.

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