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ORIGINAL RESEARCH

Resveratrol Protects PC12 Cells from High Glucose-Induced Neurotoxicity Via PI3K/Akt/FoxO3a Pathway

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Abstract Diabetes is known to be associated with neurodegenerative diseases. Resveratrol, a plant-derived polyphenolic compound found in red wine, possesses antioxidant properties. In this study, we aimed to investigate the effects of resveratrol on the phosphatidylinositol-3-kinase/protein kinase B (PI3K/Akt)/FoxO3a pathway in mediating high glucose (HG)-induced injuries in neuronal PC12 cells. PC12 cells were exposed to HG to establish a model of HG neurotoxicity. Results showed that pretreating PC12 cells with resveratrol before exposure to HG led to increased cell viability, decreased apoptotic cells, and reactive oxygen species generation. Western blot analysis showed that HG decreased the phosphorylation of Akt and FoxO3a and led to the nuclear localization of FoxO3a. These effects were significantly alleviated by resveratrol co-treatment. Furthermore, the protective effects of resveratrol were abolished by PI3K/Akt inhibitor LY294002. All these results demonstrate that resveratrol protected the PC12 cells from HG-induced oxidative stress and apoptosis via the activation of PI3K/Akt/FoxO3a signaling pathway.

Keywords Resveratrol · High glucose · Oxidative stress · Apoptosis · FoxO3a

Introduction

Glucose is the principal energy source for the mammalian brain and a substrate that is essential to maintaining normal cerebral function. A serious consequence of long-term intracellular glucose metabolism is diabetic neuropathy, which leads to nervous system damage (Tomlinson and Gardiner 2008). Hyperglycemia is the causal link in the

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evolution of neuropathy and uncontrolled diabetes (Hoeijmakers et al. 2014). Recent data have emphasized the relationship between diabetes and neurodegenerative disorders, such as Parkinson disease (PD) (Kamal et al. 2014).

Hyperglycemia is associated with elevated cellular oxidative stress (Yan 2014), which contributes to multiple organ diabetic complications, including neurodegenerative disorders (Radi et al. 2014). Oxidative stress is generated in the neural tissue when the production of free radical moieties exceeds its antioxidant capacity. Insufficient antioxidant capacity leads to free radical attack, and it damages proteins, lipids, and nucleic acids. This cellular damage triggers apoptosis in neurons and supporting glial cells, contributing to the neuropathology associated with diabetes (de la Monte and Tong 2014). Clinical evidence shows that hyperglycemia-induced oxidative stress predisposes to complications in diabetic patients, and its inhibition may block the initiation and progression of neuropathy (Arab et al. 2011).

Resveratrol is a polyphenol that is primarily found in red wine; it is known for its potent neuroprotective, anti-inflammatory, and anticarcinogenic actions (Renaud et al. 2014). Several studies showed that resveratrol has the potential to defend neurons against oxidative assaults induced by a spectrum of treatments, including neurotoxins (Wu et al. 2013; Renaud et al. 2014) and cerebral ischemic injury (Simao et al. 2012). Resveratrol plays a protective role in several neurodegenerative diseases, including PD, Alzheimer's disease (AD), and Huntington's disease (Albani et al. 2010), as well as against neuroinflammation (Foti Cuzzola et al. 2011). Notably, resveratrol protects dopaminergic PC12 cells from high glucose (HG)-induced oxidative stress and apoptosis via p53 modulation and GRP75 localization (Renaud et al. 2014). However, the underlying mechanisms of how resveratrol exerts its neuroprotective effect in HG-induced injuries are not completely elucidated.

Neuroprotective effects of resveratrol are related to the activation of the phosphatidylinositol-3-kinase/protein kinase B (PI3K/Akt) pathway (Simao et al. 2012). FoxO3a is an important downstream target of PI3K/Akt. It modulates a wide variety of cellular processes, including apoptosis, cell cycle arrest, responses to DNA damage, oxidative stress, and glucose metabolism (Monsalve and Olmos 2011). Oxidative stress can trigger FoxO3a nuclear translocation and direct affected cells to apoptosis via expression of proapoptotic gene Bim (Nakamura and Sakamoto 2008). Therefore, FoxO3a is postulated to have the potential to control cell fate in response to oxidative stress. Wilk et al. observed that HG induces significant neuronal apoptosis, accumulation of reactive oxygen species (ROS), and translocation of FoxO3a to the nucleus (2011). Recently, Li et al. found that the PI3K/Akt/FoxO3a pathway is involved in neuronal apoptosis in the developing rat brain (2009). Therefore, the PI3K/Akt/FoxO3a pathway is speculated to be involved in the protective effect of resveratrol against HG-induced injuries in PC12 cells.

To test this hypothesis, we investigated in this study the protective effects of resveratrol on HG-induced oxidative stress and apoptosis in cultured PC12 cells. The PC12 cell line, derived from a pheochromocytoma of the rat adrenal medulla, is a suitable model in investigating neuronal cell death (Martin and Grishanin 2003). The following were explored in this study: (1) the effect of HG on the phosphorylation of Akt and FoxO3a; (2) the effect of resveratrol on HG-induced increase of FoxO3a translocated to the nucleus and mediated PC12 cell death via Bim; and (3) whether resveratrol protects PC12 cells against HG-induced neurotoxicity via the PI3K/Akt/FoxO3a pathway.

Materials and Methods

Materials

Methyl thiazolyl tetrazolium (MTT), Hoechst 33258, 2′,7′-dichloro-fluorescein diacetate (DCFH-DA), D-glucose, Resveratrol, and *N*-acetyl-L-cysteine (NAC) were purchased from Sigma-Aldrich (St Louis, MO, USA). Phosphatidylinositol-3-kinase (PI3K) inhibitor LY294002 was purchased from Calbiochem. All cell culture medium components were purchased from Thermo Fisher Scientific unless otherwise noted. The enhanced chemiluminescence (ECL) solution was purchased from KeyGen Biotech (Nanjing, China).

Cell Culture

PC12 cells, a rat cell line derived from a Pheochromocytoma cells, were supplied from Sun Yat-sen University Experimental Animal Center (Guangzhou, China), and were cultured in RPMI-1640 mediums supplement with 10 % fetal bovine serum (FBS), 100 μg/ml streptomycin (Gibco, USA), and 100 U/ml penicillin streptomycin (Gibco, USA) in a humidified 5 % CO_2 atmosphere at 37 °C. PC12 cells were passaged every 2 days. PC12 cells were seeded at a density of 2×10^6 cells/dish in 100 mm dishes with 10 % calf serum incubated for 24 h and changed to 0.5 % FBS RPMI-1640 mediums for 24 h starvation. Then, PC12 cells were incubated in normal glucose (control group, 5 mM) for 24 h, high glucose (HG group, 50 mM) for 24 h, treated with resveratrol (10 μM) 30 min before high glucose for 24 h, and pretreated with LY294002 (50 μM) for 60 min before resveratrol plus high glucose.

MTT Assay

The MTT assay is a standard method used to assess cell viability. Before each experiment, PC12 cells (5,000 cells/well) were seeded in 96-well microtitre plates. PC12 cells were incubated in normal glucose (control group, 5 mM) for 24 h, high glucose (HG group, 50 mM) for 24 h, treated with resveratrol (10 μM) 30 min before high glucose for 24 h, and pretreated with LY294002 (50 μM) for 60 min before resveratrol plus high glucose. Subsequently, 10 μl MTT solution was added to each well, and the plates were incubated for 4 h at 37 °C. The absorbance was measured at 470 nm and used to calculate the relative ratio of cell viability. Three independent experiments were performed for each experimental condition.

Hoechst 33258 Nuclear Staining to Assess Apoptosis

Apoptosis was analyzed by fluorescence microscopy with the chromatin dye Hoechst 33258. After various treatments, the cells were fixed in ice-cold 4 % paraformaldehyde dissolved in phosphate-buffered saline at room temperature for 20 min. Nonspecific binding was blocked using 5 % normal goat serum in 0.01 M phosphate-buffered saline (PBS) containing 0.3 % Triton X-100 (PBS + T). Cells were washed twice with PBS and incubated with 10 μg/ml Hoechst 33258 for 15 min at room temperature in the dark. The cells were visualized under a fluorescence microscope (BX50-FLA; Olympus, Tokyo, Japan). Apoptotic cells showed condensed, fractured or distorted nuclei; viable cells displayed normal nuclear size and uniform fluorescence.

Measurement of Intracellular ROS Levels

The determination of intracellular ROS levels was performed by measuring a fluorescent product formed by the oxidation of DCFH-DA. Briefly, the culture medium was removed and the cells were washed with PBS three times. Following the addition of fresh culture medium, the cells were incubated with DCFH-DA at the final concentration of 10 μmol/l for 30 min at 37 °C. The cells were then washed again with PBS three times and the relative amount of fluorescent product was assessed using a fluorescence microscope connected to an imaging system (BX50-FLA; Olympus). The MFI from five random fields was measured using Image J 1.41o software and the MFI was used as an index of the amount of ROS. The experiment was carried out three times.

Subcellular Fractionation and Western Blot Analysis

For nuclear/cytoplasmic fractionation, cultured PC12 cells were fractionated into nuclear and cytoplasmic lysates using a PARIS kit (Ambion) according to manufacturer's instructions. Cells were homogenized directly into cell lysis buffer (Cell signaling, USA) and phosphatase inhibitor cocktail (Sigma-Aldrich), lysates were centrifugated at 14,000 rpm for 10 min at 4 °C. Protein concentration was determined with the use of a BCA protein assay kit following the manufacturer's instruction. The extracted proteins were mixed with 5 % sodium dodecyl sulfate (SDS)-PAGE sample buffer, then boiled at 100 °C for 7 min and separated by electrophoresis on a 10 % SDS-polyacrylamide gel. After electrophoresis, proteins were transferred to polyvinylidene difluoride membranes. The membranes were blocked in TBS-tween 20 (TBS-T, 0.1 % tween 20) containing 5 % non-fat dry milk for 2 h at room temperature with rotation. After blocking, the membranes were incubated with the following antibodies: rabbit anti-Akt polyclonal antibody (cell signaling 1:2,000), rabbit anti-*p*-Akt (Ser 473) monoclonal antibody (cell signaling 1:2,000), rabbit anti-FoxO3a polyclonal antibody (cell signaling 1:2,000), rabbit anti-*p*-FoxO3a (ser 253) polyclonal antibody (cell signaling 1:1,000), and rabbit anti-Bim polyclonal antibody (Abcam 1:200). Then, membranes were incubated in 5 % milk or bovine serum albumin overnight at 4 °C. Primary antibody was removed by washing the membranes three times in TBS-T, and incubated for 2 h with the appropriate horseradish peroxidase-conjugated secondary antibodies. Following 3 times of washing in TBS-T, the antigen-antibody bands were detected using enhanced chemiluminescence reagent kit and visualized using X-ray film. Each experiment was repeated three times. For quantification, the films were scanned and analyzed using Image J 1.47i software. The data of the immunoblots of phosphorylated Akt and FoxO3a were represented as a ratio of the phosphorylated forms to their total forms, respectively. The immunoblot of Bim was corrected to the bands of GAPDH.

Statistical Analysis

Results are presented as mean ± SEM. Statistical analysis of data was performed using Student's *t* test or analysis of variance (ANOVA) with SPSS 13.0 (SPSS, Chicago, IL, USA). In all cases, a difference between groups of $P < 0.05$ was accepted as statistically significant.

Results

HG Decreases the Phosphorylation of Akt and FoxO3a in PC12 Cells

To investigate the role of PI3K/Akt/FoxO3a in HG-induced toxicity, we investigated the phosphorylation of Akt and FoxO3a in PC12 cells after exposure to HG. PC12 cells were treated with HG at different times, and the phosphorylation of Akt and FoxO3a was determined by

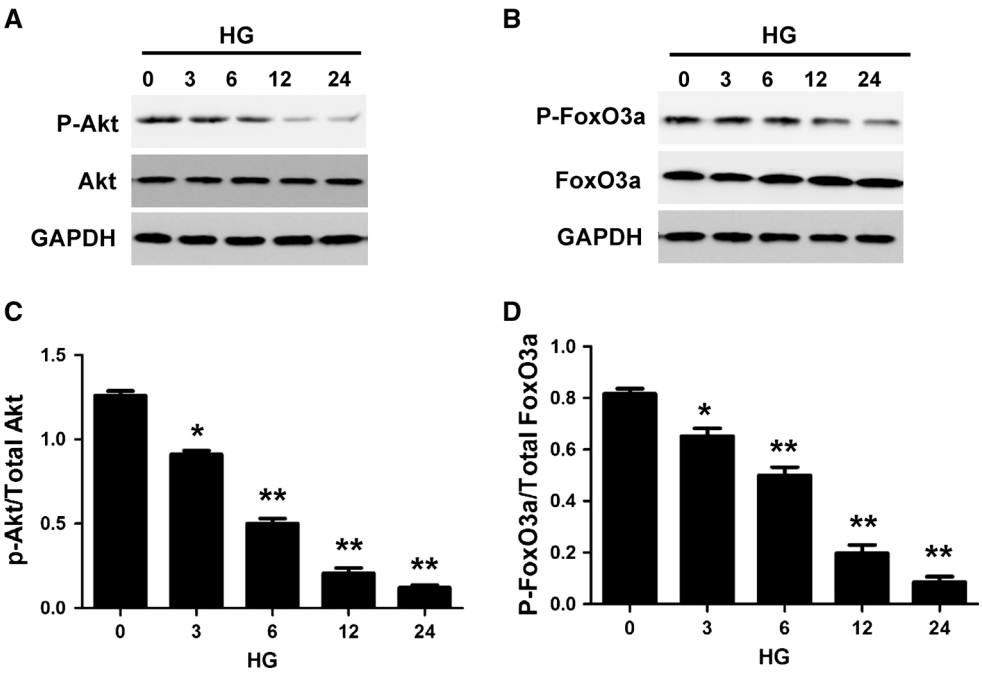

Fig. 1 HG decreases the expression of phosphorylated (*P*)-Akt and FoxO3a in PC12 cells time-dependently. PC12 cells were treated with 50 mM HG for the indicated times. **a**, **b** Phosphorylation of Akt and FoxO3a was analyzed by immunoblotting. **c**, **d** Relative levels of *p*-FoxO3a versus total FoxO3a and *p*-Akt versus total Akt in each sample as determined by blot densitometry. Data are shown as the mean ± SEM ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$ versus the control group. *HG* high glucose

Western blot. Figure 1 shows that HG decreased the phosphorylation of Akt and FoxO3a in a time-dependent manner in PC12 cells. HG induced a significant decrease in the levels of phosphorylated Akt and FoxO3a at 12 h, and it almost completely abolished the phosphorylation of Akt and FoxO3a at 24 h in PC12 cells.

Resveratrol Ameliorated HG-Induced Decreases of Phosphorylated Akt and FoxO3a in PC12 Cells

To assess whether the cytoprotective effect of resveratrol against HG-induced toxicity was associated with the PI3K/Akt/FoxO3a in PC12 cells, we detected the effect of resveratrol on the expressions of phosphorylated Akt and FoxO3a. The results showed that PC12 cell pretreatment with resveratrol for 30 min before exposure to HG for 24 h significantly increased the phosphorylated Akt and FoxO3a levels in PC12 cells compared with the HG group (Fig. 2). Furthermore, resveratrol treatment alone also significantly increased the levels of the phosphorylated Akt and FoxO3a compared with the HG-treated group. The total Akt levels and FoxO3a remained unchanged among the four groups. These findings suggest that PI3K/Akt/FoxO3a pathway may be involved in the neuroprotection of resveratrol.

NAC Ameliorated the HG-Induced Decreases of Phosphorylated Akt and FoxO3a in PC12 Cells

PC12 cells were pretreated with 1,000 μM NAC (ROS scavenger) for 60 min before exposure to HG for 24 h, to confirm whether the protective effect of resveratrol on the HG-induced decreases in phosphorylated Akt and FoxO3a is associated with antioxidation. As shown in Fig. 3, similar to the protective effect of resveratrol, cell pretreatment with NAC for 60 min markedly increased the expression of phosphorylated Akt and FoxO3a. NAC at 1,000 μM alone did not significantly change the expression of total Akt and FoxO3a. The results revealed that an antioxidant effect contributed to the protective effect of resveratrol on HG-induced injuries.

Resveratrol Induces Akt and FoxO3a Phosphorylation Via PI3K/Akt Pathways in PC12 Cells

To further establish the role of the PI3K/Akt pathway in the protective effects of resveratrol, we pretreated cells with the PI3K inhibitor LY294002 before the treatment of resveratrol plus HG. The activation of Akt and FoxO3a was determined as previously described. LY294002 abolished the stimulation of phosphorylated Akt and FoxO3a in the presence of resveratrol (Fig. 4), but elicited no effect on the expression levels of total Akt and FoxO3a. These results imply that the PI3K/Akt/FoxO3a pathway was involved in the protective effect of resveratrol.

HG Enhances Nuclear Localization of FoxO3a in PC12 Cells Whereas Resveratrol Blocks the Effect of HG

FoxO3a transcription factor functions by phosphorylation and subcellular localization. Phosphorylation of FoxO3a by Akt causes cytoplasmic localization and inhibition of its functions, such as proapoptotic effects, in neuronal cells.

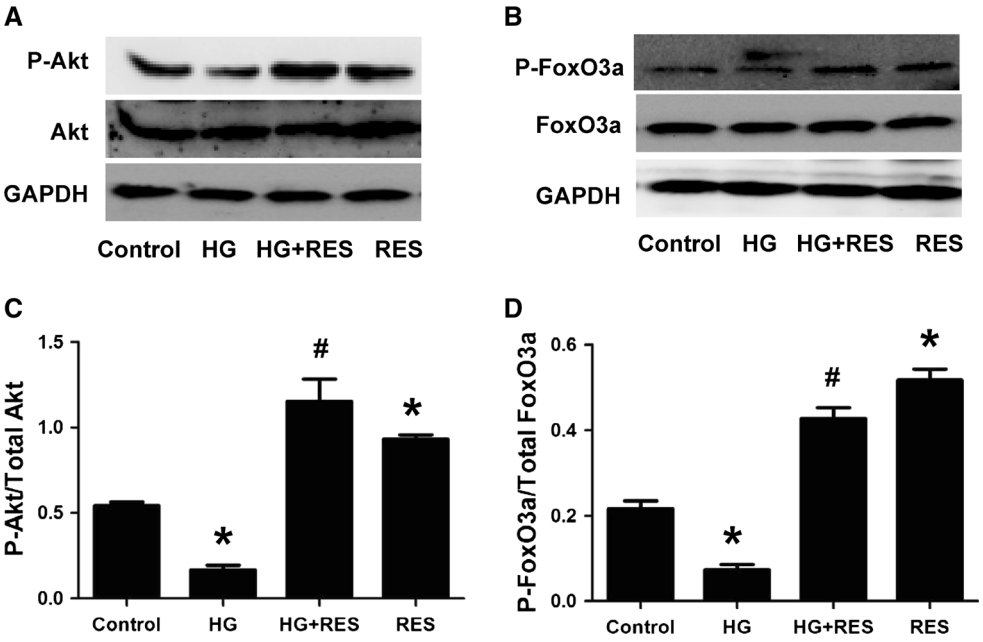

Fig. 2 Effect of resveratrol on HG-induced decreases of the expression of phosphorylated (*P*)-Akt and FoxO3a in PC12 cells. PC12 cells were treated with 50 mM HG for 24 h and either treated or pretreated with 10 μM resveratrol for 30 min. **a**, **b** Phosphorylation of Akt and FoxO3a were examined by Western blot analysis. **c**, **d** Relative levels of *p*-FoxO3a versus total FoxO3a and *p*-Akt versus total Akt in each sample as determined by blot densitometry. Data are shown as the mean ± SEM ($n = 3$). $^{*}P < 0.05$, compared with the control group; $^{\#}P < 0.05$, compared with the HG-treated group. *HG* high glucose

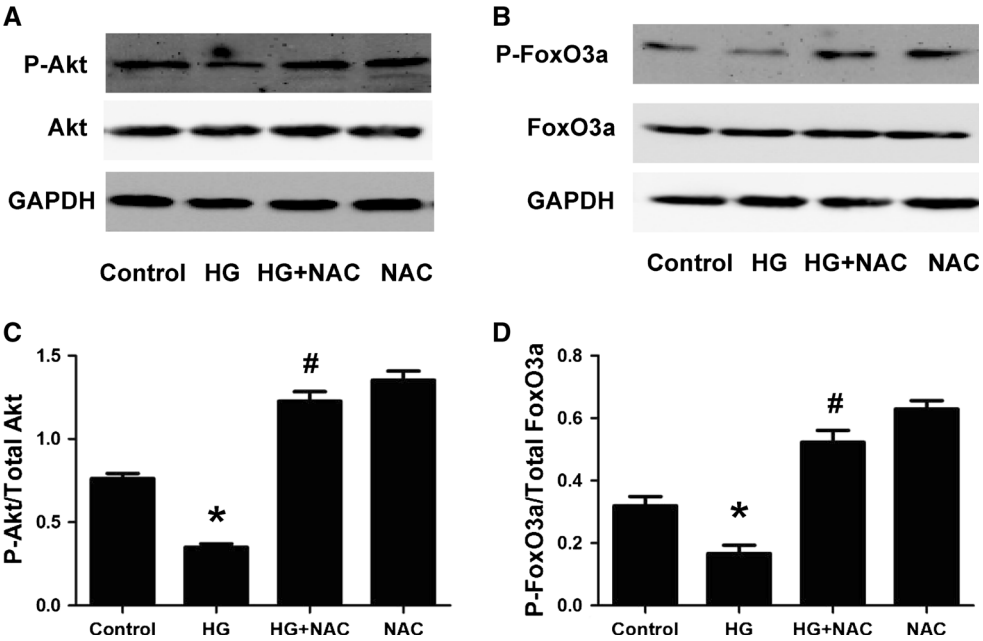

Fig. 3 Effect of NAC on HG-induced decreases of the expression of phosphorylated (*P*)-Akt and FoxO3a in PC12 cells. PC12 cells were treated with 50 mM HG for 24 h and either treated or pretreated with 1,000 μM NAC for 60 min. **a**, **b** Western blot analysis was applied to detect the change in phosphorylation of Akt and FoxO3a. **c**, **d** Relative levels of *p*-FoxO3a versus total FoxO3a and *p*-Akt versus total Akt in each sample as determined by blot densitometry. Data are shown as the mean ± SEM ($n = 3$). $^{*}P < 0.05$, compared with the control group; $^{\#}P < 0.05$, compared with the HG-treated group. *HG* high glucose

By contrast, dephosphorylation of this protein translocates FoxO3a to the nucleus and triggers apoptosis. To further investigate the effect of resveratrol and HG on FoxO3a, we studied the subcellular localization of FoxO3a following treatments with these reagents. Nuclear and cytosolic proteins from PC12 cells were extracted, and the

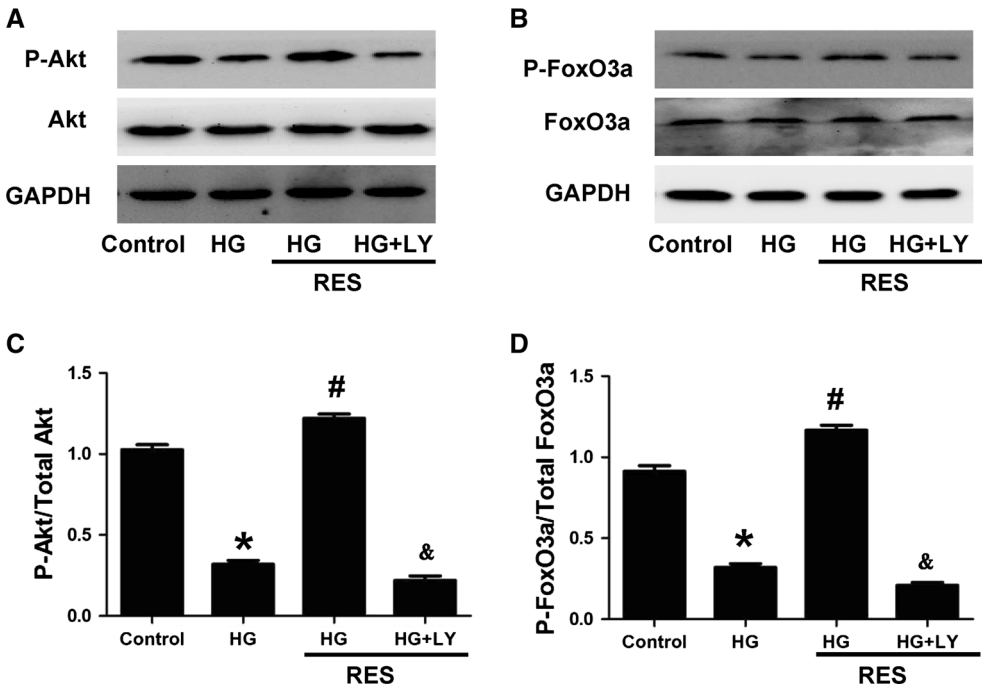

Fig. 4 LY294002 inhibited the effect of resveratrol on Akt and FoxO3a phosphorylation in PC12 cells. PC12 cells were treated with 50 mM HG for 24 h in the absence of pretreatment with 10 μM resveratrol for 30 min or 50 μM LY294002 for 60 min prior to HG exposure. **a**, **b** Phosphorylation of Akt and FoxO3a as analyzed by immunoblotting. **c**, **d** Relative levels of *p*-FoxO3a versus total FoxO3a and *p*-Akt versus total Akt in each sample as determined by blot densitometry. Data are shown as the mean ± SEM ($n = 3$). $^{*}P < 0.05$, compared with the control group; $^{\#}P < 0.05$, compared with the HG-treated group; $^{\&}P < 0.05$, compared with the RES + HG group. *HG* high glucose

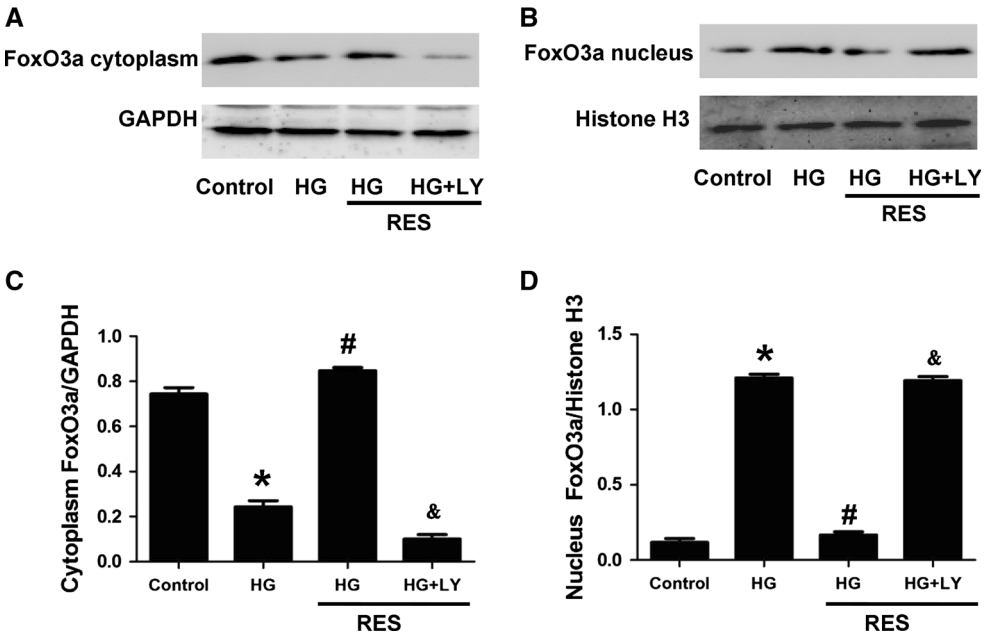

Fig. 5 LY294002 inhibited the effect of resveratrol on FoxO3a nuclear translocation. PC12 cells were treated with 50 mM HG for 24 h in the absence of pretreatment with 10 μM resveratrol for 30 min or 50 μM LY294002 for 60 min prior to HG exposure. **a**, **b** Nuclear and cytosolic protein of FoxO3a as analyzed by immunoblotting. **c**, **d** Quantification of the nuclear and cytosolic protein of FoxO3a. Data are shown as the mean ± SEM ($n = 3$). $^{*}P < 0.05$, compared with the control group; $^{\#}P < 0.05$, compared with the HG-treated group; $^{\&}P < 0.05$, compared with the RES + HG group. *HG* high glucose

subcellular localization of FoxO3a was determined. Figure 5 shows that HG enhanced the nuclear localization of FoxO3a in PC12 cells, whereas resveratrol blocked the effect of HG. Co-treatment with the PI3K inhibitor LY294002 abolished the protective effect of resveratrol.

Resveratrol Downregulates Bim Expression in a PI3K/Akt-Dependent Signaling Pathway

The downregulation of Bim protein expression was observed in the resveratrol +HG group compared with the HG group. Moreover, co-treatment with LY294002 increased Bim protein expression compared with the control group (Fig. 6). These results indicate that pretreatment with resveratrol downregulated the expression of Bim in a PI3K/Akt-dependent signaling pathway.

Resveratrol Inhibits HG-Induced Cytotoxicity

Figure 7 shows that the exposure of PC12 cells to HG for 24 h induced marked cytotoxicity, leading to a decrease in cell viability. However, cell pretreatment with 10 μM resveratrol for 30 min prior to exposure to HG significantly ameliorated the HG-induced cytotoxicity, as evidenced by an increase in cell viability. The preceding results (Figs. 2, 4) show that resveratrol ameliorated HG-induced decreases of phosphorylated Akt in PC12 cells. Thus, we aimed to confirm whether PI3K/Akt signaling pathway is involved in the protective effect of resveratrol. Pretreatment of the PC12 cells with LY294002 for 60 min prior to exposure to resveratrol plus HG abolished the protective effect of resveratrol, leading to a decrease in cell viability. Resveratrol or LY294002 alone did not alter cell viability in the PC12 cells. These findings suggest that resveratrol has a protective role in HG-induced cytotoxicity, which may involve the PI3K/Akt signaling pathway.

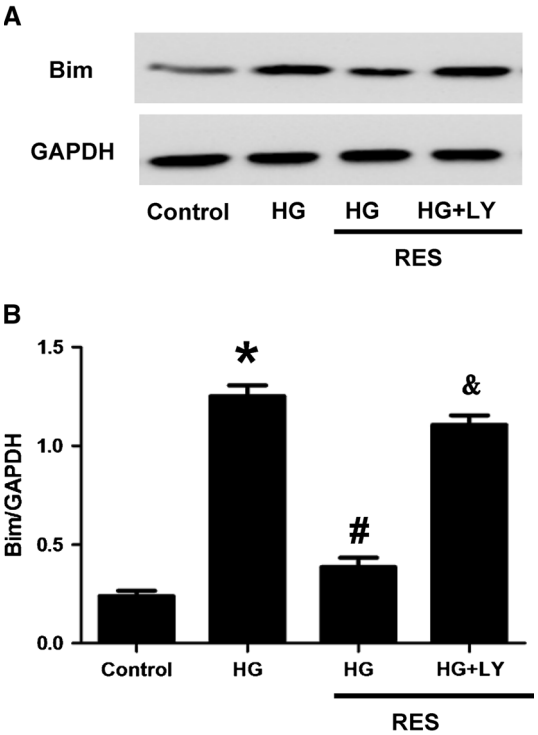

Fig. 6 LY294002 inhibited the effect of resveratrol on HG-induced Bim expression. PC12 cells were treated with 50 mM HG for 24 h in the absence of pretreatment with 10 μM resveratrol for 30 min or 50 μM LY294002 for 60 min prior to HG exposure. **a** Protein of Bim expression as analyzed by immunoblotting. **b** Relative levels of Bim in each sample as determined by blot densitometry. Data are shown as the mean ± SEM ($n = 3$). $^{*}P < 0.05$, compared with the control group; $^{\#}P < 0.05$, compared with the HG-treated group; $^{\&}P < 0.05$, compared with the RES + HG group. *HG* high glucose

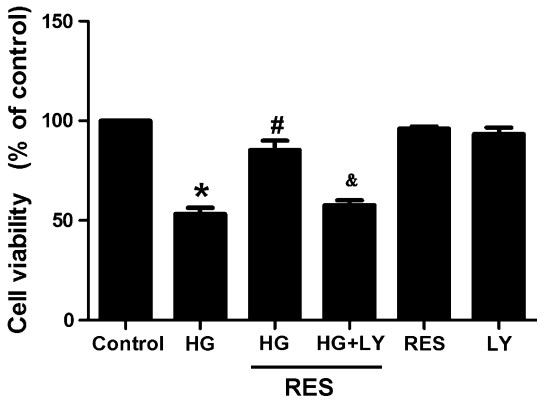

Fig. 7 Resveratrol protected PC12 cells against HG-induced cytotoxicity. Cell viability was measured by MTT assay. Data are shown as the mean ± SEM ($n = 3$). $^{*}P < 0.05$, compared with the control group; $^{\#}P < 0.05$, compared with the HG-treated group; $^{\&}P < 0.05$, compared with the RES + HG group. *HG* high glucose

Resveratrol Reduces HG-Induced Apoptosis in PC12 Cells

The effects of resveratrol on HG-induced apoptosis were observed. Figure 8 shows that the PC12 cells treated with HG for 24 h exhibited typical characteristics of apoptosis, including condensation of chromatin, shrinkage of nuclei, and apoptotic bodies. However, cell pretreatment with resveratrol for 30 min before HG exposure obviously decreased the HG-induced increased number of cells with nuclear condensation and fragmentation. Resveratrol alone did not markedly alter the cell morphology or the percentage of apoptotic PC12 cells. The aforementioned findings indicate that resveratrol protected PC12 cells against HG-induced apoptosis.

To ascertain whether PI3K/Akt signaling pathway is implicated in HG-induced apoptosis, we treated PC12 cells with LY294002 for 60 min and exposed to resveratrol plus HG. The results showed that pretreatment with LY294002

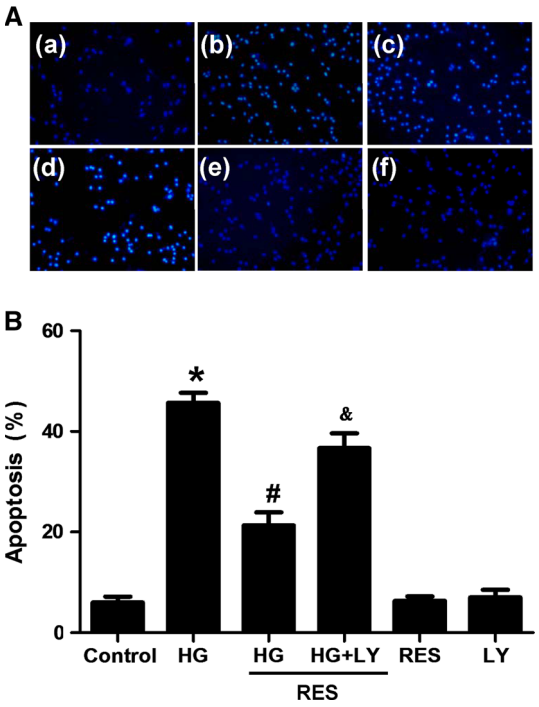

Fig. 8 Resveratrol reduced HG-induced apoptosis in PC12 cells. **a** Hoechst 33258 nuclear staining followed by fluorescence imaging to observe cell apoptosis. *a* Control group; PC12 cells were (*b*) exposed to 50 mM HG for 24 h; *c* pretreated with 10 μM resveratrol for 30 min prior to exposure to 50 mM HG for 24 h; *d* treated with 50 μM LY294002 for 60 min and 10 μM resveratrol for 30 min, followed by exposure to 50 mM HG for 24 h; *e* treated with 10 μM resveratrol for 30 min, followed by 24 h culture; *f* treated with 50 μM LY294002 for 60 min, followed by 24 h culture. **b** The apoptotic rate was analyzed with a cell counter and Image J 1.41o software. Data are shown as mean ± SEM ($n = 5$). $^{*}P < 0.05$, compared with the control group; $^{\#}P < 0.05$, compared with the HG-treated group; $^{\&}P < 0.05$, compared with the RES + HG group. *HG* high glucose

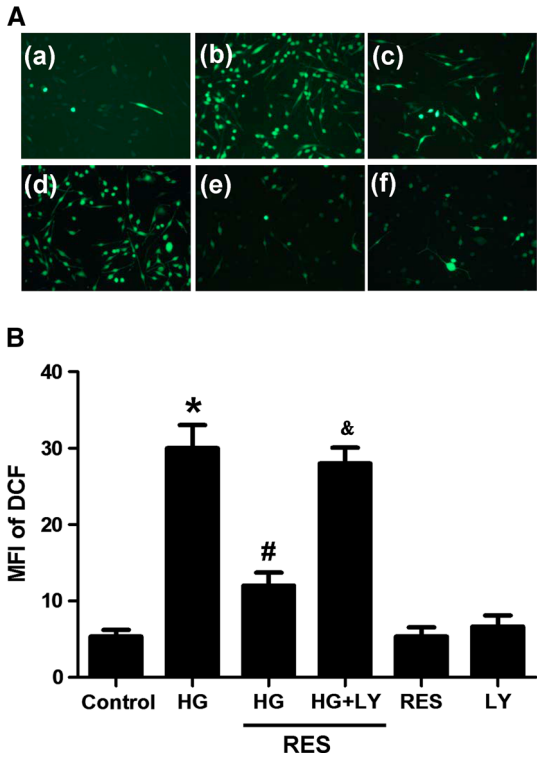

Fig. 9 Resveratrol reduced HG-induced ROS accumulation in PC12 cells. **a** After the indicated treatments, intracellular ROS generation was measured by DCFH-DA staining followed by photofluorography. *a* Control group; PC12 cells were (*b*) exposed to 50 mM HG for 24 h; *c* pretreated with 10 μM resveratrol for 30 min prior to exposure to 50 mM HG for 24 h; *d* treated with 50 μM LY294002 for 60 min and 10 μM resveratrol for 30 min, followed by exposure to 50 mM HG for 24 h; *e* treated with 10 μM resveratrol for 30 min, followed by 24 h culture; *f* treated with 50 μM LY294002 for 60 min, followed by 24 h culture. **b** Quantitative analysis of the MFI of DCF in **A** with Image J 1.41o software. Data are shown as mean ± SEM ($n = 5$). $^{*}P < 0.05$, compared with the control group; $^{\#}P < 0.05$, compared with the HG-treated group; $^{\&}P < 0.05$, compared with the RES + HG group. *HG* high glucose

abolished the protective effect of resveratrol. However, resveratrol or LY294002 alone did not induce PC12 cell apoptosis.

Resveratrol Reduces HG-Induced Oxidative Stress in PC12 Cells

The effect of resveratrol on HG-induced accumulation of ROS was tested to elucidate whether resveratrol affords neuroprotective effect against the HG-induced neuronal injury through its antioxidative action. Figure 9 shows that exposing PC12 cells to HG evidently enhanced the intracellular ROS generation. However, resveratrol preconditioning for 30 min markedly attenuated the HG-elicited ROS accumulation. Notably, pretreatment with LY294002 abolished the protective effect of resveratrol. Resveratrol or LY294002 alone did not alter the basal levels of intracellular ROS.

Discussion

Hyperglycemia is considered to be a major pathogenic factor in the development of diabetic neuropathy. However, the mechanisms associated with it have not yet been completely understood. An increasing number of research demonstrated that the stimulation of oxidative stress is critical to the evolution of metabolic syndrome, diabetes, diabetic neuropathy, and several neurodegenerative disorders, such as PD and AD (Luque-Contreras et al. 2014; Radi et al. 2014).

Hyperglycemia in diabetes causes up to fourfold increase in neuronal glucose levels. If such episodes are regular events or are persistent, then intracellular glucose metabolism leads to neuronal damage; this phenomenon is often referred to as glucose neurotoxicity (Tomlinson and Gardiner 2008). Accumulating evidence suggests that glucose

neurotoxicity is associated with the continuous ROS generation (Tomlinson and Gardiner 2008). Hyperglycemia produces ROS generation by the auto-oxidation of monosaccharides and the subsequent formation of advanced glycation end products, which in turn have direct toxic effects on nerve tissue (Hsieh and Yang 2013).

Oxidative stress is a mechanism of HG-induced neurotoxicity in diabetic patients; this mechanism leads to increased generation of ROS and apoptotic cell death (Liu et al. 2013; Cui et al. 2012). At the cellular level, the mechanisms of HG-induced neurotoxicity are similarly sustained by oxidative stress in vitro (Bournival et al. 2012; Cao et al. 2012) and in vivo (Styskal et al. 2012). This study indicates that the HG-induced cell toxicity could be mediated through ROS generation. The presence of resveratrol effectively blocked HG-induced intracellular ROS generation. Resveratrol provides protection from ROS-induced cell damage. The antioxidant and free radical scavenger's properties of resveratrol are emphasized in different studies (Renaud et al. 2014; Bournival et al. 2009).

Resveratrol potently protects against cerebral ischemia damage through the activation of the PI3K/Akt signaling pathway (Simao et al. 2012; Zhou et al. 2014). In present study, resveratrol rescued PC12 cells from the toxic effects of HG by increasing cell viability and decreasing apoptosis. The protective effect of resveratrol was completely abolished in the presence of the PI3K inhibitor LY294002. In addition, treatment of PC12 cells with resveratrol led to the rapid generation of phosphorylated Akt, which is maintained in the presence of HG. Lastly, this effect on Akt phosphorylation was blunted in the presence of LY294002. These results suggest that resveratrol promotes cell survival and protects from the toxic effects of HG by specifically activating the pro-survival PI3K/Akt pathway.

FoxO3a transcription factor is an important downstream target of PI3K/Akt pathway (Uranga et al. 2013). Activated Akt phosphorylates FoxO3a proteins at three serine/threonine residues, namely, threonine 32, serine 253, and serine 315, and leads to cytoplasmic localization of FoxO3a and the inhibition of apoptosis (Li et al. 2009). HG (Peng et al. 2013) and hypoxia (Zhang et al. 2013) decrease Akt and FoxO3a phosphorylation and induce FoxO3a activation, leading to ROS production and apoptosis in neonatal rat ventricular myocytes. Inhibition of the PI3K/Akt pathway increases the nuclear translocation of FoxO3a and promotes neuronal apoptosis (Akhter et al. 2014; Sanphui and Biswas 2013). Insulin-like growth factor-1 can induce the phosphorylation of FoxO3a by the PI3K/Akt pathway and promote the survival of hippocampal neurons and PC-12 cells (Zheng et al. 2000). Erythropoietin activates the PI3K/Akt/FoxO3a signaling pathway and protects neurons from 6-hydroxydopamine-induced apoptosis (Jia et al. 2014). In addition, Wang et al. (2013) reported that venlafaxine protects PC12 cells against corticosterone-induced cell death by modulating the activity of the PI3K/Akt/FoxO3a pathway.

To reveal the role of the PI3K/Akt/FoxO3a signaling pathway in the neuroprotective actions of resveratrol, we examined the potential role of FoxO3a in the effect of resveratrol to promote cell survival. HG inhibited the basal levels of phosphorylated FoxO3a. By contrast, resveratrol restored the levels of phosphorylated FoxO3a in the presence of HG. The action of resveratrol was abolished by pre-incubating the PI3K/Akt-specific inhibitor LY294002. Furthermore, HG caused a notable increase in the nuclear translocation of FoxO3a, resulting in an increase in Bim expression, which was prevented by resveratrol, promoting nuclear exclusion of FoxO3a and decreasing Bim expression. Taken together, these data indicate that resveratrol can mediate the survival and protection from HG-induced cell death in PC12 cells via the stimulation of the PI3K/Akt/FoxO3a signaling pathway.

In conclusion, we demonstrated for the first time that resveratrol attenuated the HG-induced oxidative stress and apoptosis via the PI3K/Akt/FoxO3a pathway in PC12 cells. These results suggest that investigating the potential of resveratrol may lead to the development of novel approaches for the prevention or possible therapy of diabetic neuropathy.

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