Allicin protects against H_2O_2-induced apoptosis of PC12 cells via the mitochondrial pathway

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Abstract. Allicin is a major bioactive ingredient of garlic and has a broad range of biological activities. Allicin has been reported to protect against cell apoptosis induced by H_2O_2 in human umbilical vein endothelial cells. The present study evaluated the neuroprotective effect of allicin on the H_2O_2-induced apoptosis of rat pheochromocytoma PC12 cells *in vitro* and explored the underlying mechanism involved. PC12 cells were incubated with increasing concentrations of allicin and the toxic effect of allicin was measured by MTT assay. The cells were pretreated for 24 h with low dose (L-), medium dose (M-) and high dose (H-) of allicin, followed by exposure to 200 μM H_2O_2 for 2 h, and the cell viability was examined by MTT assay. In addition, cell apoptosis rate was analyzed by Annexin V-FITC/PI assay, while intracellular reactive oxygen species (ROS) and mitochondrial transmembrane potential ($\Delta\psi$m) were measured by flow cytometry. Bcl-2, Bax, cleaved-caspase-3 and cytochrome *c* (Cyt C) in the mitochondria were also examined by western blotting. The results demonstrated that 0.01 μg/ml (L-allicin), 0.1 μg/ml (M-allicin) and 1 μg/ml (H-allicin) were non-toxic doses of allicin. Furthermore, H_2O_2 reduced cell viability, promoted cell apoptosis, induced ROS production and decreased $\Delta\psi$m. However, allicin treatment reversed the effect of H_2O_2 in a dose-dependent manner. It was also observed that H_2O_2 exposure significantly decreased Bcl-2 and mitochondrial Cyt C, while it increased Bax and cleaved-caspase-3, which were attenuated by allicin pretreatment. The results revealed that allicin protected PC12 cells from H_2O_2-induced cell apoptosis via the mitochondrial pathway, suggesting the potential neuroprotective effect of allicin against neurological diseases.

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Introduction

Neurological diseases, including Parkinson's, Huntington's and Alzheimer's disease, as well as traumatic brain injury and stroke, are the leading cause of mortality worldwide (1). Oxidative stress, defined as an imbalance between antioxidants and prooxidants, serves a major role in numerous biological events. The cells of the central nervous system are highly sensitive to injuries induced by oxidative stress (2). Increasing evidence has revealed that apoptosis, inflammation and oxidative stress are correlated with these neurological diseases (3-5). Reactive oxygen species (ROS), including hydrogen peroxide (H_2O_2), hydroxyl radical ($HO\cdot$) and superoxide radical (O_2^-), are byproducts of cellular processes and are primarily generated in the mitochondrion of the cells (6,7). Approximately 1-2% of the mitochondrial oxygen consumption is used to produce ROS (8). ROS are reported to serve a critical role in the release of pro-apoptotic proteins and cytochrome *c* (Cyt C), which activate caspase family members and induce cell apoptosis (9). Therefore, inhibiting oxidative stress-induced neuronal injury is considered as a therapeutic strategy in the treatment of neurological diseases (10).

Garlic possesses various biological properties, such as immunomodulatory, anticancer, antiaging, antimicrobial, antihypertensive and antiatherosclerotic effects (11-14). These properties are closely correlated with the bioactive ingredients of garlic (15). Allicin is the main compound extracted from garlic and has strong antioxidant activity (16,17). Chen *et al* have demonstrated that allicin protects human umbilical vein endothelial cells (HUVECs) from H_2O_2-induced cell apoptosis by inhibiting oxidative stress (18).

In the present study, H_2O_2 was used to establish an *in vitro* model of oxidative stress injury, and the intervention effect of allicin on the apoptosis of rat pheochromocytoma PC12 cells was evaluated. To the best of our knowledge, this is the first to evaluate the effect of allicin on H_2O_2-induced apoptosis of PC12 cells *in vitro*.

Materials and methods

Cell culture. Rat pheochromocytoma PC12 cells were obtained from the Cell Bank at the Chinese Academy of Sciences (Shanghai, China). PC12 cells were maintained in RPMI 1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; GE Healthcare Life Sciences, South Logan, Utah, USA). The cells were cultured in a 5% CO_2 incubator at 37˚C.

Cytotoxicity of allicin. PC12 cells were cultured in 96-well plates ($6x10^3$ cells/well) for 24 h and incubated with increasing concentrations of allicin (0, 0.01, 0.1, 1, 10, 100 or 1,000 μg/ml; Yuanye Bio-Technology Co., Ltd., Shanghai, China) for a further 24 h. MTT (5 mg/ml, 20 μl; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was added into each well and cultured at 37˚C for 4 h to produce formazan crystals. After discarding the medium, the cells were treated with dimethyl sulfoxide (Sigma-Aldrich), and the absorbance was analyzed at 490 nm (BioTek Instruments, Inc., Winooski, VT, USA). Concentrations of allicin found to be non-toxic were selected for subsequent experiments.

Cell treatment. After culturing for 24 h, PC12 cells were pretreated with 0.01 (low dose, L-allicin), 0.1 (medium dose, M-allicin) or 1 μg/ml (high dose, H-allicin) of allicin for 24 h and then exposed to 200 μM hydrogen peroxide (H_2O_2, Xilong Chemical Co., Ltd., Shenyang, China) for 2 h. Cells incubated only with 200 μM H_2O_2 for 2 h served as the H_2O_2 group, while untreated cells served as the control.

Cell viability. Subsequent to incubation with H_2O_2 and allicin, the cell viability was determined by MTT (Sigma-Aldrich; Merck KGaA) assay as previously described (19). The absorbance of cells was detected with a microplate reader (BioTek Instruments, Inc.) at 490 nm.

Cell apoptosis. AnnexinV-FITC/propidium iodide (PI) assay (catalogue no. WLA001b; Wanleibio, Shenyang, China) was performed to analyze cell apoptosis. Briefly, PC12 cells were washed with phosphate-buffered saline (PBS; Shanghai Double-helic Biology Science and Technology Co., Ltd., Shanghai, China) and resuspended in binding buffer (500 μl), followed by incubation with Annexin V-FITC (5 μl) and PI (5 μl) in the dark. After washing twice with PBS, the cells were collected and cell apoptosis was analyzed by a BD Accuri™ C6 flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). LL represents survival cells (Annexin V^-/PI^-). LR represents early apoptotic cells (Annexin V^+/PI^-). UR represents late apoptotic or necrotic cells (Annexin V^+/PI^+). UL represents dead cells (Annexin V^-/PI^+). The total apoptotic cell rate was calculated as follows: Early apoptotic cell rate + late apoptotic cell rate.

Measurement of ROS level. Intercellular ROS level was measured according to the protocol of the Reactive Oxygen Species Assay kit (catalogue no. S0033; Beyotime Institute of Biotechnology, Haimen, China). Briefly, DCFH-DA (10 mM) supplied in the kit was diluted to 10 μM with serum-free medium. Subsequent to the indicated allicin and H_2O_2 treatment, the medium was discarded, and PC12 cells were incubated with the diluted DCFH-DA (2 ml) at 37˚C for 20 min and washed three times with serum-free medium. Subsequently, the cells were washed twice with PBS and detected by flow cytometry (BD Biosciences) to determine the ROS levels.

Measurement of mitochondrial transmembrane potential ($\Delta\psi m$). The $\Delta\psi m$ was determined using a JC-1 Apoptosis Detection kit (catalogue no. KGA601; KeyGen Biotech Co., Ltd., Nanjing, China) according to the manufacturer's instructions. Briefly, the cells were collected following the indicated treatment and resuspended in 500 μl incubation buffer containing 1 μl JC-1 at 37˚C for 20 min. Subsequent to centrifugation (550 x g for 5 min at room temperature), the cells were washed and resuspended in 1X incubation buffer (KeyGen Biotech Co., Ltd.). The cells were then subjected to flow cytometry (BD Biosciences) in order to determine the $\Delta\psi m$. UR represents normal cells. LR represents early apoptotic cells.

Western blotting. The cells were cultured in 6-well plates at a density of $4x10^5$ cells/well prior to being lysed in lysis buffer (Wanleibio) on ice and total proteins were obtained by centrifugation (10,005 x g for 10 min at 4˚C). The cells were homogenized and mitochondrial proteins were isolated using a Mitochondrial Protein Extraction kit (catalogue no. WLA034; Wanleibio) according to the manufacturer's instructions. Protein concentration was measured using a BCA kit (catalogue no. WLA004; Wanleibio). Subsequently, 40 μg protein was separated by 7, 10 or 12% SDS-PAGE and transferred onto polyvinylidene fluoride membranes (EMD Millipore, Bedford, MA, USA). After blocking with non-fat milk for 1 h, the membranes were incubated with polyclonal antibodies against Bcl-2 (WL01556; 1:500 dilution; Wanleibio), Bax (WL01637; 1:500 dilution; Wanleibio), cleaved-caspase3 (WL01992; 1:500 dilution; Wanleibio), Cyt C (WL01571; 1:500 dilution; Wanleibio), COX IV (WL01794; 1:500 dilution; Wanleibio) and β-actin (WL01845; 1:1,000 dilution; Wanleibio). β-actin and COX IV served as the internal controls for total proteins and mitochondrial proteins, respectively. Subsequently, cells were incubated with goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (WLA023; 1:5,000 dilution; Wanleibio). The protein bands were visualized using an enhanced chemiluminescence reagent (Wanleibio) and quantified with Gel-Pro-Analyzer version 4.0 software (Media Cybernetics, Bethesda, MD, USA).

Statistical analysis. Results are expressed as the mean ± standard deviation. Statistical analysis was performed by Student's t test or one-way analysis of variance followed by Bonferroni's multiple comparison test using GraphPad Prism 5 (GraphPad Software, Inc., San Diego, CA, USA). Differences with a $P<0.05$ were considered as statistically significant.

Results

Selection of the non-toxic concentrations of allicin. The cytotoxicity of various concentrations allicin was determined by MTT assay. As shown in Fig. 1, the allicin doses of 10 ($P<0.05$), 100 ($P<0.01$) and 1,000 μg/ml ($P<0.01$) significantly decreased

the viability of PC12 cells compared with the untreated cells. However, the other three concentrations of allicin (0.01, 0.1 and 1 μg/ml) did not markedly affect the cell viability. Therefore, these three doses were named as the low (L-allicin; 0.01 μg/ml), medium (M-allicin; 0.1 μg/ml) and high dose groups (H-allicin; 1 μg/ml).

Allicin attenuates H_2O_2-induced cell growth inhibition. To assess the protective effect of allicin on the cell proliferation of PC12 cells, the cells were pretreated with L-allicin, M-allicin or H-allicin for 24 h and then incubated with H_2O_2. Cell viability was determined by MTT assay. As shown in Fig. 2, H_2O_2 (200 μM) significantly impaired the cell viability of PC12 cells (P<0.01). However, allicin treatment markedly improved the decreased cell viability caused by H_2O_2 in a dose-dependent manner, with the medium and high doses having a significant effect (both P<0.01).

Allicin protects PC12 cells against H_2O_2-induced cell apoptosis. Annexin V-FITC/PI assay was performed to evaluate the effect of allicin on H_2O_2-induced cell apoptosis. As shown in Fig. 3, incubation with H_2O_2 significantly increased the apoptosis rate to 24.43±2.07% compared with the control group (2.94±0.45%; P<0.01). However, allicin treatment significantly lowered the apoptosis rate in the L-allicin, M-allicin and H-allicin groups to 18.72±2.50, 6.87±1.03 and 6.15±0.47%, respectively, compared with the rate in the H_2O_2 group (P<0.05, P<0.01 and P<0.01, respectively).

Allicin restores ROS level and Δψm in H_2O_2-treated PC12 cells. The study further evaluated the effect of allicin on H_2O_2-induced ROS generation using DCFH-DA (Fig. 4A-E). The intracellular ROS levels in the control and H_2O_2-treated cells were 8.27±1.26 and 34.39±2.77%, respectively. By contrast, the ROS levels in the allicin-treated cells were 28.03±2.70, 17.73±1.86 and 11.11±1.68%, respectively. These results showed that H_2O_2 treatment significantly elevated the intracellular ROS level (P<0.01; Fig. 4F). Notably, pretreatment with allicin inhibited H_2O_2-induced ROS production in a dose-dependent manner (L-allicin, P<0.05; M-allicin, P<0.01; H-allicin, P<0.01).

In order to determine the Δψm, PC12 cells were stimulated with H_2O_2 for 2 h and then stained with JC-1 prior to flow cytometric analysis (Fig. 5A-E). The results demonstrated that H_2O_2 exposure resulted in the loss of Δψm compared with the control group (P<0.01; Fig. 5F). However, allicin prevented the loss of Δψm in H2O2-stimulated PC12 cells in a dose-dependent manner (M-allicin, P<0.01; H-allicin, P<0.01).

Effect of allicin on the expression of Bcl-2, Bax, cleaved-caspase-3 and mitochondrial Cyt C. The levels of Bcl-2, Bax, cleaved-caspase-3 and mitochondrial Cyt C were examined by western blotting subsequent to allicin and H_2O_2 treatment. As shown in Fig. 6, H_2O_2 exposure greatly decreased Bcl-2 and mitochondrial Cyt C levels, whereas it increased Bax and cleaved-caspase-3 levels when compared with the control group (P<0.01). Allicin pretreatment reversed the effect of H_2O_2 on the expression of Bcl-2 (M-allicin, P<0.05; H-allicin, P<0.01), Bax (H-allicin, P<0.01), cleaved-caspase-3 (M-allicin, P<0.05; H-allicin, P<0.01) and mitochondrial Cyt C (H-allicin, P<0.01).

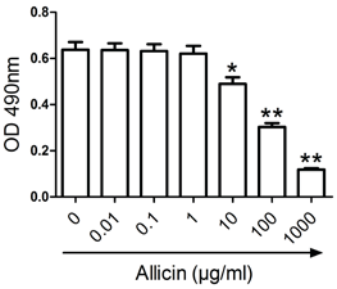

Figure 1. Cytotoxicity of various concentrations of allicin (0, 0.01, 0.1, 1, 10, 100 or 1,000 μg/ml) in PC12 cells incubated for 24 h. Cell viability was examined by MTT assay. *P<0.05 and **P<0.01 vs. the control group.

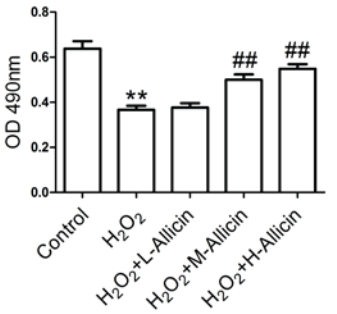

Figure 2. Effect of allicin on cell viability. PC12 cells were pretreated with L-allicin (0.01 μg/ml), M-allicin (0.1 μg/ml) and H-allicin (1 μg/ml) for 24 h and then exposed to H_2O_2 for 2 h. MTT assay was conducted to determine the cell viability. **P<0.01 vs. the control group; ##P<0.01 vs. the H_2O_2 group.

Discussion

Allicin, an active compound extracted from garlic, has antitumor, anti-inflammatory, anti-oxidative and anti-microbial activities (20,21). Oxidative stress serves a vital role in the neurodegeneration process, and H_2O_2 has been reported to be an inductor of ROS release, which contributes to the occurrence and progression of neurodegenerative diseases (22). Previous studies have observed that allicin exerts neuroprotective effects against traumatic brain injury *in vitro* and *in vivo* via the Akt/eNOS signaling pathway due to its anti-oxidative and anti-inflammatory activities (23,24). In addition, allicin administration alleviates learning and memory impairment in a mice model of Alzheimer's disease by inhibiting the p38 MAPK pathway (25).

To the best of our knowledge, the present study was the first to evaluate the neuroprotective effect of allicin in H_2O_2-stimulated rat pheochromocytoma PC12 cells. The effect of increasing concentrations of allicin on PC12 cell viability was evaluated, and three relative low concentrations of allicin (0.01, 0.1 and 1 μg/ml) were selected for further experiments. Next, the neuroprotective effect of allicin on cell viability, apoptosis, ROS generation, Δψm and the mitochondrial intrinsic pathway were further evaluated in H_2O_2-treated PC12 cells.

H_2O_2 treatment has been commonly used as a method to evaluate antioxidant efficiency or oxidative stress

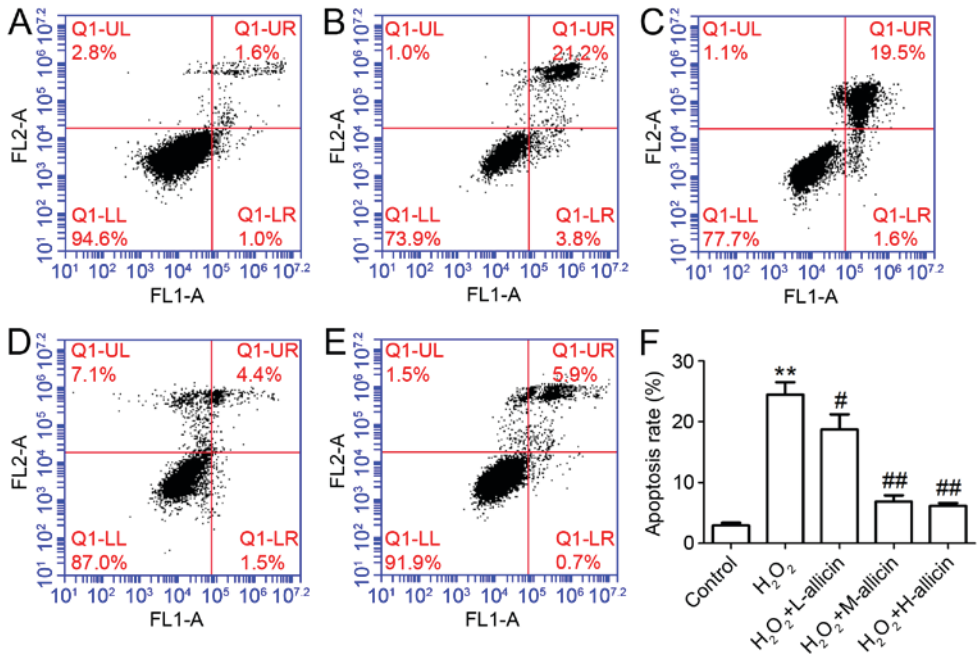

Figure 3. Effect of allicin on cell apoptosis. Following treatment with allicin and H_2O_2, cell apoptosis was analyzed by Annexin V-FITC/propidium iodide staining. Flow cytometry findings of (A) control, (B) H_2O_2, (C) H_2O_2+L-allicin, (D) H_2O_2+M-allicin and (E) H_2O_2+H-allicin are presented. (F) Quantified results of apoptosis rate. **P<0.01 vs. the control group; $^{\#}$P<0.05 and $^{\#\#}$P<0.01 vs. the H_2O_2 group. L-allicin, 0.01 μg/ml; M-allicin, 0.1 μg/ml; H-allicin, 1 μg/ml.

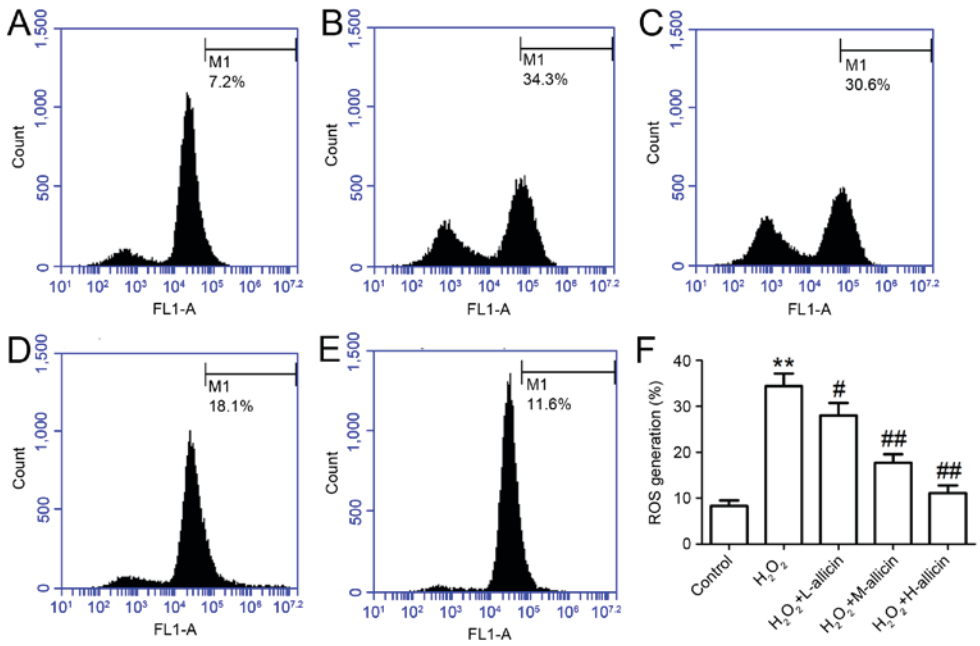

Figure 4. Effect of allicin on H_2O_2-induced ROS generation. PC12 cells were pretreated with allicin for 24 h, followed by exposure to H_2O_2 for 2 h. The ROS levels were measured using DCFH-DA and flow cytometry. Flow cytometry findings of (A) control, (B) H_2O_2, (C) H_2O_2+L-allicin, (D) H_2O_2+M-allicin and (E) H_2O_2+H-allicin are presented. (F) Quantified results of ROS generation. **P<0.01 vs. the control group; $^{\#}$P<0.05 and $^{\#\#}$P<0.01 vs. the H_2O_2 group. L-allicin, 0.01 μg/ml; M-allicin, 0.1 μg/ml; H-allicin, 1 μg/ml; ROS, reactive oxygen species.

susceptibility of cells that are susceptible to oxidative injury (26). In the present study, we established an *in vitro* model of H_2O_2-induced oxidative injury in PC12 cells. It was observed that H_2O_2 promoted PC12 cell apoptosis, increased intracellular ROS levels, reduced $\Delta\psi$m, decreased mitochondrial Cyt C levels and Bcl-2 levels, and elevated Bax and cleaved-caspase-3 levels. These findings were in agreement with previous reports (27,28).

The degeneration of neurons in the brain or spinal cord is associated with neurodegenerative disease (29). In the present study, the effect of allicin on cell viability in the present of H_2O_2 was firstly investigated. It was demonstrated that allicin attenuated the inhibitory effect of H_2O_2 on cell proliferation in a dose-dependent manner. Apoptosis is a process of programmed cell death that is regulated by the extrinsic pathway and the intrinsic pathway (30). It has been

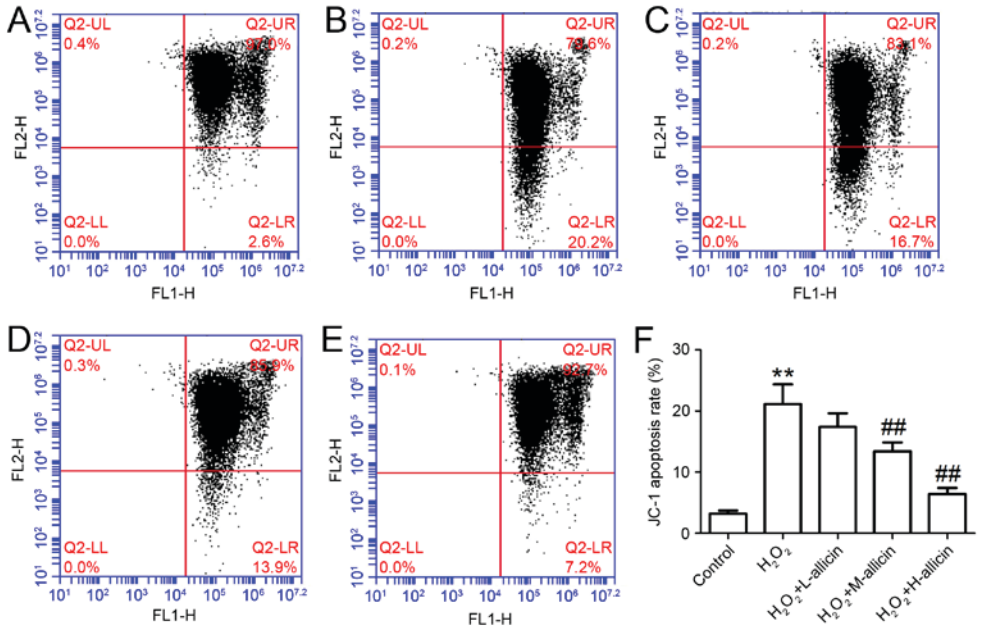

Figure 5. Effect of allicin on Δψm. After 24-h incubation in 6-well plate, the cells were subjected to H_2O_2 and allicin treatment. The Δψm of PC12 cells was analyzed by flow cytometry. Flow cytometry findings of (A) control, (B) H_2O_2, (C) H_2O_2+L-allicin, (D) H_2O_2+M-allicin, and (E) H_2O_2+H-allicin are presented. (F) Quantified results of Δψm. **P<0.01 vs. the control group; $^{\#\#}$P<0.01 vs. the H_2O_2 group. L-allicin, 0.01 μg/ml; M-allicin, 0.1 μg/ml; H-allicin, 1 μg/ml; Δψm, mitochondrial transmembrane potential.

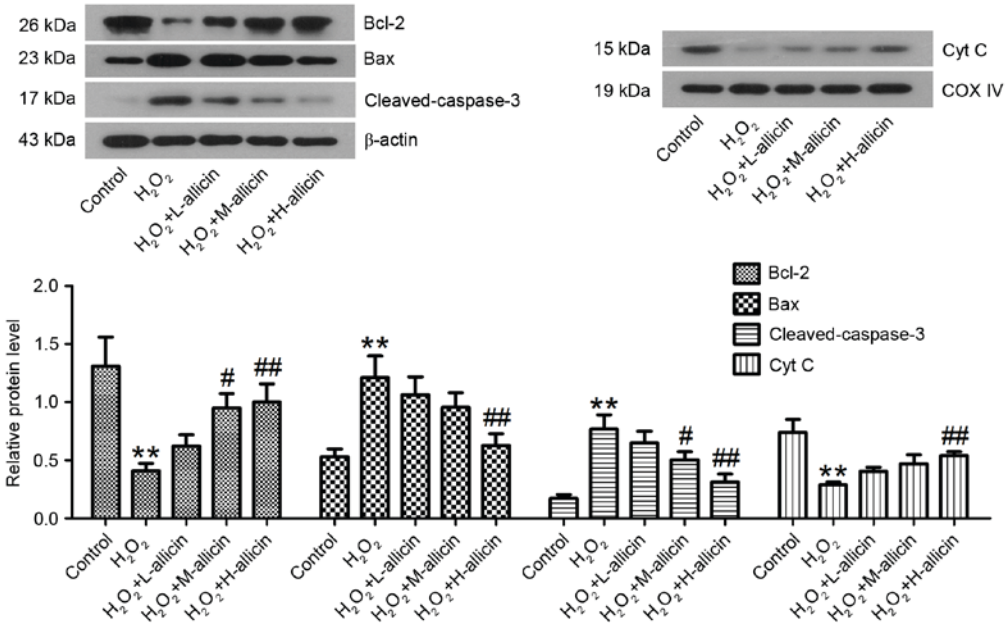

Figure 6. Effect of allicin on the expression of various mitochondrial pathway regulators. Subsequent to treatment, total proteins and mitochondrial proteins were extracted from the PC12 cells. The levels of Bax, Bcl-2, cleaved-caspase-3 and Cyt C in the mitochondria were quantified by western blot analysis. β-actin and COX IV served as the internal controls for total proteins and mitochondrial proteins, respectively. **P<0.01 vs. the control group; $^{\#}$P<0.05 and $^{\#\#}$P<0.01 vs. the H_2O_2 group. L-allicin, 0.01 μg/ml; M-allicin, 0.1 μg/ml; H-allicin, 1 μg/ml; Cyt C, cytochrome *c*.

reported that allicin suppressed the apoptosis of rat H9c2 cells and HUVECs induced by H_2O_2 (18,31). In the present study, the results of Annexin V-FITC/PI assay showed that H_2O_2 markedly promoted PC12 cell apoptosis, which was inhibited by allicin pretreatment in a dose-dependent manner. These findings indicate that allicin protected H_2O_2-treated PC12 cells by increasing cell viability and inhibiting cell apoptosis.

ROS functions in multiple intracellular signaling pathways as a secondary messenger and serves as a mediator in inflammation and oxidative injury (2). Mitochondria are the major ROS-producing organelle and the target of ROS (32). Apoptosis signals initially lead to the enhancement of mitochondrial permeability and the loss of Δψm. Cyt C is then translocated into the cytosol and caspase-3/9 is activated to induce cell apoptosis (33,34). The depolarization of Δψm,

mitochondria swelling, the increase of malondialdehyde and ROS, and the decrease of superoxide dismutase are the characteristics of mitochondrial dysfunction (35). Zhu *et al* have demonstrated that allicin provides protection against spinal cord ischemia/reperfusion injury in rabbits through enhancing antioxidant enzyme activities and improving mitochondrial function (36). Furthermore, allicin alleviated H_2O_2-induced cytotoxicity in retinal pigmented epithelial cells by reducing ROS and oxidative stress (37). The current study results consistently demonstrated that allicin ameliorated H_2O_2-induced ROS generation and the collapse of $\Delta\psi m$ in rat pheochromocytoma PC12 cells. The results indicate that allicin protected against H_2O_2-induced apoptosis by inhibiting the dysfunction of mitochondria.

The expression of Bcl-2 family members, Bcl-2 (an anti-apoptotic protein) and Bax (a pro-apoptotic protein), are known to be associated with the apoptotic process. In addition, the imbalance between Bcl-2 and Bax results in the release of Cyt C from the mitochondria, which in turn activates the downstream caspases (38,39). Caspases include various cysteine proteases that are responsible for cell apoptosis in eukaryotes (40). Caspase-3, also known as a molecular switch, belongs to the cysteine protease family and degrades downstream substrates during apoptosis (41). The results of the present study demonstrated that allicin reversed the effect of H_2O_2 on the expression of Bcl-2, Bax, cleaved-caspase-3 and mitochondrial Cyt C in PC12 cells (18), suggesting the involvement of the mitochondrial pathway in the neuroprotection of PC12 cells.

In conclusion, allicin protected PC12 cells against H_2O_2-induced oxidative injury via the mitochondrial pathway. The present study provides evidence for the clinical application of allicin as a candidate anti-oxidative drug for neuroprotection.

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