Allicin protects against H$_2$O$_2$-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$_2$O$_2$ in human umbilical vein endothelial cells. The present study evaluated the neuroprotective effect of allicin on the H$_2$O$_2$-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$_2$O$_2$ 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$_2$O$_2$ reduced cell viability, promoted cell apoptosis, induced ROS production and decreased $\Delta\psi_m$. However, allicin treatment reversed the effect of H$_2$O$_2$ in a dose-dependent manner. It was also observed that H$_2$O$_2$ 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$_2$O$_2$-induced cell apoptosis via the mitochondrial pathway, suggesting the potential neuroprotective effect of allicin against neurological diseases.

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$_2$O$_2$), hydroxyl radical (HO$_2$) 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$_2$O$_2$-induced cell apoptosis by inhibiting oxidative stress (18).

In the present study, H$_2$O$_2$ 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$_2$O$_2$-induced apoptosis of PC12 cells in vitro.

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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% CO₂ incubator at 37°C.

Cytotoxicity of allicin. PC12 cells were cultured in 96-well plates (6x10⁴ 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 (H₂O₂; Xilong Chemical Co., Ltd., Shenyang, China) for 2 h. Cells incubated only with 200 µM H₂O₂ for 2 h served as the H₂O₂ group, while untreated cells served as the control.

Cell viability. Subsequent to incubation with H₂O₂ 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 H₂O₂ 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.

Western blotting. The cells were cultured in 6-well plates at a density of 4x10⁴ 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. WAL034; 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). 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 H₂O₂-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 H₂O₂. Cell viability was determined by MTT assay. As shown in Fig. 2, H₂O₂ (200 µM) significantly impaired the cell viability of PC12 cells (P<0.01). However, allicin treatment markedly improved the decreased cell viability caused by H₂O₂ in a dose-dependent manner, with the medium and high doses having a significant effect (both P<0.01).

**Allicin protects PC12 cells against H₂O₂-induced cell apoptosis.** Annexin V-FITC/PI assay was performed to evaluate the effect of allicin on H₂O₂-induced cell apoptosis. As shown in Fig. 3, incubation with H₂O₂ 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 9.12±0.75, 6.87±0.63 and 6.15±0.47%, respectively, compared with the rate in the H₂O₂ group (P<0.05, P<0.01 and P<0.01, respectively).

**Allicin restores ROS level and ∆ψm in H₂O₂-treated PC12 cells.** The study further evaluated the effect of allicin on H₂O₂-induced ROS generation using DCFH-DA (Fig. 4A-E). The intracellular ROS levels in the control and H₂O₂-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 H₂O₂ treatment significantly elevated the intracellular ROS level (P<0.01; Fig. 4F). Notably, pretreatment with allicin inhibited H₂O₂-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 ∆ψm, PC12 cells were stimulated with H₂O₂ for 2 h and then stained with JC-1 prior to flow cytometric analysis (Fig. 5A-E). The results demonstrated that H₂O₂ exposure resulted in the loss of ∆ψm compared with the control group (P<0.01; Fig. 5F). However, allicin prevented the loss of ∆ψm 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 H₂O₂ treatment. As shown in Fig. 6, H₂O₂ 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 H₂O₂ 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).

**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 H₂O₂ has been reported to be an inducer 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 H₂O₂-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, ∆ψm and the mitochondrial intrinsic pathway were further evaluated in H₂O₂-treated PC12 cells.

H₂O₂ treatment has been commonly used as a method to evaluate antioxidant efficiency or oxidative stress
susceptibility of cells that are susceptible to oxidative injury (26). In the present study, we established an in vitro model of H_{2}O_{2}-induced oxidative injury in PC12 cells. It was observed that H_{2}O_{2} promoted PC12 cell apoptosis, increased intracellular ROS levels, reduced δψ, 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_{2}O_{2} was firstly investigated. It was demonstrated that allicin attenuated the inhibitory effect of H_{2}O_{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 3. Effect of allicin on cell apoptosis. Following treatment with allicin and H_{2}O_{2}, cell apoptosis was analyzed by Annexin V-FITC/propidium iodide staining. Flow cytometry findings of (A) control, (B) H_{2}O_{2}, (C) H_{2}O_{2}+L-allicin, (D) H_{2}O_{2}+M-allicin and (E) H_{2}O_{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_{2}O_{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_{2}O_{2}-induced ROS generation. PC12 cells were pretreated with allicin for 24 h, followed by exposure to H_{2}O_{2} for 2 h. The ROS levels were measured using DCFH-DA and flow cytometry. Flow cytometry findings of (A) control, (B) H_{2}O_{2}, (C) H_{2}O_{2}+L-allicin, (D) H_{2}O_{2}+M-allicin and (E) H_{2}O_{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_{2}O_{2} group. L-allicin, 0.01 µg/ml; M-allicin, 0.1 µg/ml; H-allicin, 1 µg/ml; ROS, reactive oxygen species.
reported that allicin suppressed the apoptosis of rat H9c2 cells and HUVECs induced by H$_2$O$_2$ (18,31). In the present study, the results of Annexin V-FITC/PI assay showed that H$_2$O$_2$ markedly promoted PC12 cell apoptosis, which was inhibited by allicin pretreatment in a dose-dependent manner. These findings indicate that allicin protected H$_2$O$_2$-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 $\Delta\psi_m$. Cyt C is then translocated into the cytosol and caspase-3/9 is activated to induce cell apoptosis (33,34). The depolarization of $\Delta\psi_m$,
In conclusion, allicin protected PC12 cells against H₂O₂-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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