Neuroprotective Effect of Ginkgolide K against H$_2$O$_2$-Induced PC12 Cell Cytotoxicity by Ameliorating Mitochondrial Dysfunction and Oxidative Stress

Shuwei Ma,*a Xingyan Liu,b Qingrui Xunc, and Xiantao Zhangd

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The authors declare no conflict of interest.

* To whom correspondence should be addressed. e-mail: mashuwei_2004@163.com.

Materials Ginkgolide K was extracted and separated by our laboratory, its purity was more than 98% and its sample...
was deposited at Institute of Traditional Chinese Medicine of Guangdong Province. Its chemical constitution was shown in Fig. 1. PC12 cells (ATCC, CRL-1721) were gifted by New Drug Research Center of China Pharmaceutical University (Nanjing, China). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), RPMI 1640 medium, fetal bovine serum, horse serum, H₂O₂, penicillin, streptomycin and 5,5′,6,6′-tetrachloro-1,1′,3′,3′-tetraethylbenzimidazolocarbocyanine iodide (JC-1) all were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Dimethyl sulfoxide (DMSO) was bought from Changzhou Chemical Corp. (Changzhou, China). Lactate dehydrogenase (LDH) assay kit, ROS assay kit, Annexin V/Propidium iodide (PI) apoptosis assay kit, Hoechst 33342, caspase-3 activity kit, caspase-8 activity kit, caspase-9 activity kit and cytochrome c antibody were obtained from Kaji Institute of Biological Engineering (Nanjing, China). Nitrocellulose membrane and enhanced chemiluminescence (ECL) Western detection reagent was purchased from Amersham Bioscience (Piscat-away, NJ, U.S.A.). All other reagents were from Sigma-Aldrich (St. Louis, MO, U.S.A.) unless otherwise stated.

**Cell Culture and Treatment** PC12 cells were cultured in RPMI 1640 medium supplemented with 5% (v/v) fetal bovine serum, 5% (v/v) horse serum, 100 U/mL penicillin, and 100 U/mL streptomycin (Lunarp Pharmaceutical Co., Ltd., Jinan, China) at 37°C in a humidified atmosphere of 5% CO₂. Cells were passaged into poly-L-lysine-coated cell culture flask at 1×10⁵ cells/cm² and differentiated by treating with 100ng/mL nerve growth factor for 1 week as described previously. After cultured with the serum-free medium for 24h, the differentiated PC12 cells were pretreated with various concentrations (10, 50 or 100µM) of ginkgolide K for 24h prior to exposure to H₂O₂ (0.3 mM) for 24h. Control cells were not treated with ginkgolide K or H₂O₂. H₂O₂ injury cells were treated with H₂O₂ (0.3 mM) for 24h alone. All measurements were performed after the cells were exposed to H₂O₂ for 24h.

**Cell Viability Assay** Cell viability was measured by using the MTT assay that was based on the conversion of MTT to formazan crystals by mitochondrial dehydrogenases. In order to observe the influence of ginkgolide K on the viability of PC12 cells, PC12 cells (1×10⁵ cells/cm²) were treated with the different concentrations of ginkgolide K from 1µM to 100µM for 24h at 37°C. Besides, to test a dose- and time-dependencies of H₂O₂-treatment in PC12 cells, PC12 cells were harvested and fixed with 4% paraformaldehyde for 20min at room temperature, then were washed 3 times with 0.02 M PBS and exposed to 10 mg/mL Hoechst 33342 for 30min at room temperature in the dark. The samples were observed under a fluorescence microscope (Olympus Imaging America Inc., Center Valley, PA 18034–0610, U.S.A.) at 200× magnification.

**Flow Cytometry (FCM) Analysis for Apoptosis** The apoptotic cells were quantitated by Annexin V/propidium iodide (PI) apoptosis assay kit. After treatment, PC12 cells were trypsinized, resuspended in binding buffer and stained with Annexin V/PI for 15min. Samples were analyzed by FACScan flow cytometer with an excitation wavelength of 488nm and an emission wavelength of 530nm (Becton-Dickinson, Bedford, MA, U.S.A.). FL1 represented Annexin V fluorescence while FL2 showed PI fluorescence. At least 10000 events were recorded. Apoptotic cells were expressed as a percentage of the total number of cells.

**Cellular Permeability Analysis by HPLC** To test the cellular permeability of ginkgolide K, cells were treated with ginkgolide K at 100µM. After 4, 8 or 24h incubation, cells were washed with ice cold Tris-buffered saline (pH 7.4) and harvested in 0.15 mL ice-cold lysis PBS buffer. The cell slurry was transferred into a microcentrifuge tube, and then vortexed followed by sonication for 5s. Intracellular ginkgolide K was extracted by centrifugation at 15000rpm for 20min at 4°C. An aliquot (0.1 mL) of the supernatant was immediately collected and filtered into the HPLC autosampler. Twenty micro-
liter of each sample was injected into the Agilent 1200 series HPLC system (U.S.A.) equipped with an Agilent XDB-C18 column (4.6 mm i.d. × 250 mm, 5 mm) at a flow rate of 1.0 mL/min using 40% water and 60% methanol as the mobile phase with a detection wavelength of UV 225 nm.

**Measurement of Intracellular ROS** Intracellular ROS was monitored by using the 2′,7′-dichlorofluorescin diacetate (DCFH-DA) fluorescent probe. Intracellular H$_2$O$_2$ or low-molecular-weight peroxides can oxidize DCFH-DA to dichlorofluorescin (DCF) with the highly fluorescent absorption. At the end of treatment, cells were incubated with 10 mM DCFH-DA at 37°C for 30 min, and then washed twice with PBS. Finally, the fluorescence intensity of DCF was measured in a microplate reader with an excitation wavelength of 485 nm and an emission wavelength of 535 nm.

**Measurement of MMP** Cells were treated with ginkgolide K and H$_2$O$_2$ as described above. Cells were incubated in Hank’s solution containing 10 mg/mL JC-1 for 30 min at 37°C and then were centrifuged for 5 min at 2000 rpm. Cells sample was collected and washed twice with pre-warmed Hank’s solution (37°C) followed by re-floating and analyzing with microplate reader (Rato company, U.S.A.) at an excitation wavelength of 488 nm and an emission wavelength of 535 nm. The mean fluorescence intensity (MFI) in the cells represented the state of depolarization of MMP.

**Measurement of Cytochrome c Release** The release of cytochrome c from mitochondria into the cytosol was assessed by Western Blot. Briefly, after the cells were processed as the above procedure, cells were collected and centrifuged at 1000 rpm for 10 min. Subsequently, cells were washed twice with PBS and resuspended on lysis buffer consisting of 75 mM NaCl, 1 mM Na$_2$HPO$_4$, 8 mM Na$_2$HPO$_4$, 250 mM sucrose supplemented with 200 mg digitonin/mL and 1:100 of a broad spectrum protease inhibitor cocktail (Sigma Chemical Co.). After vortexing this suspension was centrifuged at 15000 rpm for 5 min at 4°C and the supernatant, corresponding to the cytosolic fraction, was recovered. Seventy-five micrograms of cytosolic protein extract was loaded onto each lane of sodium dodecyl sulfate (SDS)-polyacrylamide gel. The separated proteins were electrotransferred to a polyvinylidene difluoride membrane (Amersham Pharmacia Biotech, Buckinghamshire, U.K.). The membranes were blocked for 2 h at room temperature with blocking buffer (15 mM NaCl, 2.5 mM Tris–HCl, pH 7.6, and 0.1% Tween-20) containing 5% non-fat dry milk. The blots were then incubated with primary mouse monoclonal antibody against the denatured form of cytochrome c (1:400) for 12 h. The membranes were then washed four times with PBS for 30 min and incubated with an alkaline phosphatase-conjugated antibody to mouse immunoglobulin (Ig) (1:3000) for 2 h at room temperature. The membranes were washed five times with PBS for 30 min. The bands were revealed with ECF (Lingqi company limited, Wuxi, China) and visualised by using a Versa-Doc Imaging System, Model 3000 (BioRad, Hercules, CA, U.S.A.). The images were analyzed with the Quantity One software (BioRad). β-Actin was used as a loading control. Protein concentrations were quantified by the BioRad protein assay dye while bovine serum albumin was employed as the standard.

**Measurement of Caspase Activity** At the end of experiments, the cells were harvested and washed twice with PBS. Then, cells were resuspended on an ice lysis buffer consisting of 1 mM Na-ethylenediaminetetraacetic acid (Na-EDTA), 1 mM Na-ethylene glycol–bis(b-aminoethyl ether)–N,N-tetraacetic acid (Na-EGTA), 2 mM MgCl$_2$, 6H$_2$O, 25 mM N-(2-hydroxyethyl)perazine-N’-2-ethanesulfonic acid (HEPES) (pH 7.5) supplemented with 0.1% CHAPS, 100 mM phenylmethlysulfonyl fluoride (PMSF), 2 mM dithiothreitol (DTT), and 1:100 of a broad spectrum protease inhibitor cocktail (Sigma Chemical Co.). After freezing during three times in liquid nitrogen, the cell extracts were centrifuged at 20000 rpm for 10 min at 4°C and the supernatants were stored at −70°C. To measure caspase activity, aliquots of lysates containing 25 mg of protein were added to a reaction buffer (25 mM HEPES, pH 7.5) supplemented with 0.1% CHAPS, 5 mM DTT and 100 mM PMSF. The reactions were initiated after addition of the following fluorimetric substrates (50 mM final concentration): Ac-DEV-D-Amc for caspase-3-like activity, Ac-IETD-Amc for caspase-8-like activity and Ac-LEDH-Afc for caspase-9-like activity.
activity. After 2 h of incubation at 37°C the cleavage of the substrates was measured (Amc, excitation 390 nm and emission 475 nm; Afc, excitation 400 nm and emission 505 nm) with a microplate reader.23)

Statistical Analyses Data were presented as the mean±standard deviation (S.D.). Statistical analysis was conducted using one-way ANOVA followed by a post hoc LSD test. p<0.05 was considered to indicate statistical significance. The SPSS software package (SPSS program, version 13.0) was used for all statistical tests.

RESULTS

Effects of Ginkgolide K on PC12 Cell Viability Compared with control group, the viability of PC12 cells treated with gingolide K alone at the difference concentrations from 1 µM to 100 µM was not significantly increase or decreased with the viability of 102.1±6.2%, 104.3±7.0%, 99.6±5.2% of control, respectively. As shown in Fig. 1B.

Cytotoxicity of H2O2 on PC12 Cell PC12 cells (1×10⁵ cells/mL) were treated H2O2 at different concentration at 37°C for 24 h. MTT assay showed that the cell viability was inhibited by H2O2 in a concentration-dependent manner with an IC₅₀ of 0.3 mM of (Fig. 2), thus H2O2 (0.3 mM) was chosen for the subsequent experiments.

Effects of Ginkgolide K on H2O2-Induced PC12 Cell Viability Compared with control group, the cell viability was significantly decreased to 49.6±4.3% after treatment with H2O2 (0.3 mM) alone for 24 h. However, treated PC12 cells with the different ginkolide K (10 µM, 50 µM, 100 µM) prior to H2O2 damage would result in the marked increase of cell viability to 57.9±5.7, 70.4±4.6, 79.9±5.3% of control as shown in Fig. 3A.

Effects of Ginkgolide K on LDH Leakage The release of LDH from PC12 cells in control group was 162.2±28.9 U/L, The release of LDH from PC12 cells treated with H2O2 (0.3 mM) for 24 h was increased to 520.2±40.9 U/L. However, pretreatment with various concentrations of ginkolide K (10 µM, 50 µM, 100 µM) could significantly decrease the LDH leakage to 463.7±32.7 U/L, 304.8±34.2 U/L, 238.3±21.2 U/L, respectively. Therefore, the results demonstrated that ginkolide K could inhibit LDH leakage from PC12 cells in a concentration-dependent manner (Fig. 3B).

Effect of Ginkgolide K on the Morphology of Cell Nuclei As shown in Fig. 4A, after H2O2 administration, the
Fig. 5. Effect of Ginkgolide K on H₂O₂-Induced PC12 Apoptosis by Annexin V Staining (Flow Cytometry Analysis)

PC12 cells were pretreated with ginkgolide K (10, 50 and 100 µM) for 24 h and then exposed to 0.3 mM H₂O₂ for 24 h. Cell apoptosis was tested by flow cytometry analysis. Results were obtained from three independent experiments and were expressed as mean±S.D. **p<0.01 vs. control group without H₂O₂, *p<0.05 vs. group treated with H₂O₂ alone.

Fig. 6. Determination of Incorporation into PC12 Cell of Ginkgolide K

PC12 cells were treated with ginkgolide K (100 µM) in six holes culture plate. After incubation for 4, 8 and 24 h, 100 µL intracellular extracts were aliquoted, and 20 µL of each sample was subjected to HPLC analysis. HPLC chromatograms were shown by blank culture medium (A), intracellular ginkgolide K at 4 h (B), and intracellular ginkgolide K at 8 h (C) and intracellular ginkgolide K at 24 h (D), respectively.
PC12 cells revealed that nuclear of apoptosis cell transformed into light blue fluorescence contrasted to viable cell with faint blue fluorescence. The apoptotic cells of PC12 cells treated with 0.3 mM H$_2$O$_2$ for 24 h increased to 55.7±6.7% than control cells (7.7±1.6%). Pretreatment with different concentration of ginkgolide K (10, 50, and 100 µM) reduced the apoptotic cells in a concentration-dependent manner from 47.1±5.9% and 39.5±4.9% to 25.5±4.8%, respectively (Fig. 4B).

**Effect of Ginkgolide K on Apoptosis** To quantitatively demonstrate the effect of ginkgolide K in H$_2$O$_2$-induced apoptosis, Annexin V/PI staining was measured by flow cytometric analysis. As presented in Figs. 5A and B, the 9.8±1.1% of total cells in the control group was apoptosis. However, Treatment of cells with 0.3 mM H$_2$O$_2$ alone significantly increased apoptotic rate to 51.7±6.3%. Pre-incubation with ginkgolide K (10, 50 and 100 µM) markedly reduced the number of cells labeled with Annexin V/PI$^-$ and Annexin V$^+$/PI$^-$, which percentage of apoptosis cells was significantly decreased to 42.8±2.8%, 27.7±4.4%, 10.9±1.8%, respectively.

**Effect of Ginkgolide K on Cell Permeability** Intracellular level of ginkgolide K was analyzed by HPLC to determine the permeability of ginkgolide K across the membrane barrier in PC12 cells. Figure 6 showed that the exposure to 100 µM of ginkgolide K for 4, 8 and 24 h resulted in the increase of ginkgolide K in cytosol to 0.8, 12 and 24 µM, respectively. This result suggested that ginkgolide K was possessed of partial membrane permeability *in vitro*.

**Effect of Ginkgolide K on Intracellular ROS Content** As shown in Fig. 7, treatment of PC12 cells with 0.3 mM H$_2$O$_2$ for 24 h led to an increase in DCF fluorescence with a 2.8-fold increment than control. However, pretreatment with ginkgolide K effectively reduced ROS generation and the suppressing effect gradually was strengthened with the increasing concentrations of ginkgolide K. Ginkgolide K at different concentration (10, 50, 100 µM) lessened H$_2$O$_2$-induced ROS production from 247.2±22.4% and 176.2±24.7% to 144.8±19.2%.

**Effect of Ginkgolide K on MMP Expression** After incubation of PC12 cells with H$_2$O$_2$ (0.3 mM) for 24 h, the MMP expression was decreased to 60.2±5.9% of control (Fig. 8). Pretreatment with different concentration of ginkgolide K (10, 50 and 100 µM) protected cells against the H$_2$O$_2$-induced reducing of MMP expression from 68.7±5.1% and 79.8±9.7% to 88.4±5.6%.

**Effect of Ginkgolide K on Cytochrome c Release** Level of cytochrome c in cytosol was increased to 468.5±36.4% compared with control group when the cells were treated with H$_2$O$_2$ alone. However, the cells were pretreated with ginkgolide K for 24 h, the level of cytochrome c were decreased to 396.4±44.4% (100 µM), 279.5±37.8% (50 µM) and 246.4±24.4% (10 µM), respectively (Fig. 9).

**Effect of Ginkgolide K on Caspase Activation** Compared with the control group, caspase-9-like activity was increased 2.5-fold after treated with 0.3 mM H$_2$O$_2$ for 24 h. Furthermore, caspase-3 was significantly activated in cells treated with 0.3 mM of H$_2$O$_2$ for 24 h (5.8-fold of control value). However, after cells were incubated with various concentrations of ginkgolide K (10, 50, 100 µM) for 24 h prior to exposure to H$_2$O$_2$, the caspase-9 and -3-like activity were decreased to 1.4- and 1.5-fold, 1.9- and 1.6-fold, 2.9- and 4.1-fold than the control, respectively. Additionally, caspase-8-like activity was increased in H$_2$O$_2$-induced cell (1.2-fold of control value).
value). However, caspase-8-like activity in cells pre-treated with the different concentration ginkgolide K was not significantly altered compared with H2O2 (Fig. 10).

DISCUSSION

Exposure PC12 cells to H2O2 significantly decreased the cell viability with the same results from Hoechst 33342 positive cells and apoptosis rate, and increased the LDH release from cells. However, ginkgolide K pretreatment in PC12 cells remarkably increased the cell viability and lessened the LDH release, the results implied that ginkgolide K observably protected PC12 cells against H2O2-induced cytotoxicity.

Ginkgolide K was a liposoluble compound. Thus, we investigated whether it smoothly passed through cytomembrane when PC12 cells were incubated with ginkgolide K (100 µM) from 4h to 24h in vitro. The intracellular concentrations of ginkgolide K in PC12 cells were determined using high performance liquid chromatography methods. The results illustrated that the majority of ginkgolide K were prevented from entering PC12 cells even at 24h after incubation, which implied that the protective effect of ginkgolide K on H2O2-induced damage to PC12 cells was mainly provided with a small quantity of ginkgolide K incorporating into PC12 cells. Therefore, we legitimately concluded that the elevation in penetrability of ginkgolide K could obviously produce the stronger protective action than our present results in H2O2-induced PC12 cells.

It is now clear that the mitochondria are located at the centre of the cell death regulation process. A number of genes and proteins can influence or determine the progression of apoptosis along the mitochondrial pathway. The change of mitochondrial membrane permeability could induce the release of cytochrome c, which could consequently activate caspase-3 apoptosis protein, then lead to DNA ladder. Therefore, the variation of MMP and the release of cytochrome c from mitochondria in PC12 cells were detected to illustrate the protective mechanism of mitochondrial pathway. In the present study, we found that treatment of differentiated PC12 cells with H2O2 (0.3 mM) for 24h led to cell injury and apoptosis as shown in Fig. 3, which was accompanied by MMP loss and the cytochrome c release from mitochondria. However, when the cells were treated with ginkgolide K prior to expose to H2O2, the elevation of cytochrome c release from mitochondria and the loss of MMP were ameliorated to impede the apoptotic protein activation, the results showed that the protective effect of ginkgolide K was associated with reduction of mitochondria membrane permeability caused by MMP downgrade and indirectly blocking the release of apoptosis factor including cytochrome c.

ROS (superoxide, hydroxyl radicals, and peroxynitrite) are produced during ischemic injury due to alteration in mitochondrial respiration. ROS have significant cellular effects including lipid peroxidation, protein denaturation, inactivation of enzymes, nucleic acid and DNA damage, release of Ca2+ from intracellular stores, damage to the cytoskeletal structure and chemotaxis. On the other hand, MMP loss causes the release of apoptosis-inducing factors and generates secondary ROS, and, as a result, ultimately leads to cell death. The research displayed that H2O2 led to ROS production while ginkgolide K-pretreated significantly attenuated ROS level. Sequentially, the inhibition of cytochrome c release from mitochondria via pretreatment with ginkgolide K can attribute to the elimination of ROS.

Caspases play an important role in the apoptotic process in two ways: the death receptor pathway and the mitochondrial pathway. No matter which pathway is involved, caspase-3 acts as an apoptotic executor. Capase-3 activates DNA fragmentation factor, which in turn activates endonucleases to cleave nuclear DNA and ultimately leads to cell death. In the mitochondrial pathway, a variety of stimuli triggers the mitochondrial permeability transition and the release of cytochrome c, then activation of caspase-3. Inhibition of caspase-3 has shown promising effect in attenuating apoptosis. In the present study, we found that H2O2 remarkably increase caspase-3 activity. Whereas, pre-incubation with ginkgolide K depressed
the caspase-3 activity. In addition, activation of caspase-9 activity was generally thought to follow the disruption of the outer mitochondrial membrane, which caused a collapse of membrane potential and a change of permeability. 35) The previous study showed that the cytochrome c released from mitochondrion combined with caspase-9 precursor and resultantly led to activation of caspase-9 activity. 36) In our experiment, the result implied that exposure of PC12 cells to H2O2 significantly increased caspase-9 activity. However, pretreatment with ginkgolide K reversed the elevation of caspase-9 activity. Meanwhile, the major pathway of caspase-8 activation, known as extrinsic apoptotic pathway, was considered to be based on death receptor, which in turn promoted dimerization and subsequent activation of this caspase. 37) However, there were some evidences suggesting that caspase-8 could also be activated by intrinsic triggers independent of the death receptor pathway in both in vivo and in vitro paradigms. 38–37) Caspase-8 activation has also been observed in various cell types during apoptosis in response to H2O2. 39) Consistent with these reports, our experiment results showed that H2O2 could lead to the enhancement of caspase-8 activity. However, pretreatment with ginkgolide K prior to exposure to H2O2 did not inhibit caspase-8 activity, implying that the protective effect of ginkgolide K in PC12 cells apoptosis induced by H2O2 was not related to inhibition of caspase-8 activity. Thus, the anti-apoptotic mechanism of ginkgolide K is possibly attributed to mitochondria-regulated apoptosis channels including decrease of the cytochrome c delivery, increase of MMP and inhibition of caspase-9, caspase-3 activity.

CONCLUSION

In summary, in differentiated PC12 cells treated with H2O2, apoptosis was associated with MMP change, ROS promotion, and consequently caspase-3 activation. This report suggested that H2O2 induced apoptosis in PC12 cells by decreasing MMP and elevating ROS level, which resulted in cytochrome c release and caspase-3 activation. However, ginkgolide K could protect PC12 cells from H2O2-induced apoptosis through increasing MMP, relieving ROS level and ultimately inhibition caspase-3 activity. Therefore, ginkgolide K may be a potential neuroprotective compound for protecting the neuron injury induced by H2O2, the further study was needed to elucidate its underlying molecular mechanism.

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