Protective effect of hopeahainol A, a novel acetylcholinesterase inhibitor, on hydrogen peroxide-induced injury in PC12 cells

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ABSTRACT

In this study, we evaluated the effects of hopeahainol A, a novel acetylcholinesterase inhibitor (AChEI) from *Hopea hainanensis*, on H_2O_2-induced cytotoxicity in PC12 cells and the possible mechanism. Exposure of PC12 cells to 200 μM H_2O_2 caused cell apoptosis, reduction in cell viability and antioxidant enzyme activities, increment in malondialdehyde (MDA) level, and leakage of lactate dehydrogenase (LDH). Pretreatment of the cells with hopeahainol A at 0.1–10 μM before H_2O_2 exposure significantly attenuated those changes in a dose-dependent manner. Moreover, hopeahainol A could mitigate intracellular accumulation of reactive oxygen species (ROS) and Ca^{2+}, the loss of mitochondrial membrane potential (MMP), and the increase of caspase-3, -8 and -9 activities induced by H_2O_2. These results show that hopeahainol A protects PC12 cells from H_2O_2 injury by modulating endogenous antioxidant enzymes, scavenging ROS and prevention of apoptosis. There was potential for hopeahainol A to be used in treating Alzheimer's disease (AD) that involved acetylcholinesterase, free radical, oxidative damage and cell apoptosis.

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1. Introduction

Alzheimer's disease (AD), characterized by a progressive loss of cognitive function, is a neurodegenerative disorder. The epidemiological studies concluded that there were 25 million AD patients worldwide in 2000, and this number would be over 114 million by 2050 if there is no new preventive or neuroprotective therapies to be established (Wimo et al., 2003). Acetylcholinesterase inhibitors (AChEIs) are the mainstays for the treatment of AD and have been part of standard care (Cummings, 2004). Four AChEIs, tacrine, donepezil, rivastigmine and galantamine, have been so far approved for the treatment of AD. Though beneficial in improving cognitive and behavioral symptoms, the effectiveness of AChEIs has been questioned since they do not delay or prevent neurodegeneration (Small, 2005; Terry and Buccafusco, 2003). Increasing evidence supports the role for oxidative damage in the pathogenesis of AD and neuronal degeneration of AD patients is associated with oxidative damage to all biomacromolecule types (Lovell and Markesbery, 2007; Sayre et al., 2008). These implicated that the brains of AD patients are being challenged by intensified oxidative stress (Behl, 1999; Lustbader et al., 2004). Therapeutic strategies aimed at removal of free radicals or prevention of their formation might be a reasonable choice for the management of the disease. So antioxidants have been considered as a potential treatment of AD (Lleó et al., 2006).

Hopeahainol A (Fig. 1) was an AChE inhibitory polyphenol with a new skeleton isolated from *Hopea exalata* in our laboratory (Ge et al., 2008). Since some polyphenolic compounds were disclosed to be antioxidative and neuroprotective (Frade et al., 2005), we hypothesized that hopeahainol A could be both AChE-inhibitory to alleviate symptoms of AD, and antioxidant to protect the neurons from the ROS elicited neurodegeneration. We therefore examined the protective effects of hopeahainol A on H_2O_2-induced cytotoxicity in cultured PC12 cells which were widely used for neurobiological and neurochemical studies (Denes et al., 2006; Li et al., 2008; Zhang et al., 2008).

2. Materials and methods

2.1. Materials

Hopeahainol A was isolated from *Hopea exalata*, and its structure was described previously (Ge et al., 2008). The purity of the compound was more than 98% by high-performance liquid chromatography (HPLC) analysis. Phenol red free RPMI 1640 medium was purchased from HyClone (Hyclone, UT, USA). The kits used for LDH, MDA and glutathione peroxidase (GSH-Px) assays were obtained from Jiancheng Bioengineering Institute (Nanjing, China). Caspase activity assay kits were purchased from Beyotime Institute of Biotechnology (Nantong, Jiangsu, China). Propidium iodide (PI), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Hoechest 33258, DCFH-DA and Rhodamine 123 were purchased from Sigma–Aldrich Inc. (St. Louis, MO, USA). Fura 2-AM was purchased from Dojindo Laboratories (Kumamoto, Japan). All other chemicals were of analytic grade.

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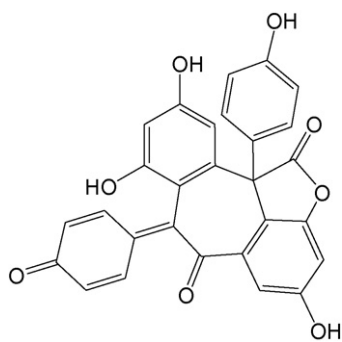[1] These authors equally contributed to the work.

Fig. 1. Structure of hopeahainol A.

2.2. Cell culture and experimental treatment

PC12 cells (adrenal gland pheochromocytoma) were high passages from American Type Culture Collection (ATCC) and maintained at 37 °C in the humidified atmosphere containing 5% CO_2 in phenol red free RPMI 1640 medium, supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine. All experiments were carried out 24 h after the cells were seeded onto plates or dishes. Hopeahainol A was freshly prepared as stock solution in dimethylsulfoxide (DMSO) and diluted with phosphate-buffered saline (PBS) before the experiment. To produce oxidative stress, H_2O_2 was freshly prepared from 30% stock solution prior to each experiment. PC12 cells were pre-incubated with hopeahainol A for 30 min before H_2O_2 was added. Assays for cell viability, LDH activity, MDA, CAT activity, GSH-Px activity, apoptosis, ROS, Ca^{2+}, MMP and caspase activities were performed at different time after H_2O_2 was added.

2.3. Analysis of cell viability

Cell viability was evaluated by the reduction of MTT. Briefly, after PC12 cells (2×10^4 cells/ml) were treated with 200 μM H_2O_2 in the presence of hopeahainol A for 6 h at 37 °C, MTT solution in phosphate-buffered saline was added to the medium with the final concentration of 0.5 mg/ml. After 4 h incubation with MTT, cells were lysed in dimethyl sulfoxide and the amount of MTT formazan was quantified by determining the absorbance at 570 nm using a microplate reader (SPECTRAFLUOR, TECAN, Sunrise, Austria). Cell viability was expressed as a percent of the control culture value.

2.4. Analysis of LDH

After PC12 cells were exposed to 200 μM H_2O_2 for 6 h, the medium was collected, and the amount of LDH released by cells was determined using an assay kit according to the manufacturer's protocol. The absorbance of samples was read at 440 nm.

2.5. Analysis of MDA, GSH-Px and CAT

After exposed to 200 μM H_2O_2 for 6 h, PC12 cells were washed with ice-cold PBS, harvested by centrifugation at $1000 \times g$ for 5 min, pooled in 0.5 ml of 100 mM phosphate buffer (pH 7.4) and homogenized. The homogenate was centrifuged at $3000 \times g$ for 20 min at 4 °C. The resulting supernatant was stored at −20 °C until MDA and enzyme activities were assayed. Protein content was measured according to Bradford (1976), using bovine serum albumin (BSA) as the standard.

The content of MDA and the GSH-Px activity were determined by using assay kits according to the manufacturer's protocol.

CAT activity was measured according to the method of Sun et al. (2005) in 50 mM PBS (pH 7.0) using 50 mM H_2O_2 as substrate. The decomposition of H_2O_2 was monitored by measuring the change in absorbance at 240 nm for 3 min. One unit of activity corresponds to the loss of 1 μM H_2O_2 per min.

2.6. Hoechst staining

After exposed to 200 μM H_2O_2 for 12 h, PC12 cells growing on 24-well plates were fixed with 4% paraformaldehyde for 30 min at room temperature, then washed with pre-chilled PBS three times and exposed to 1 μg/ml Hoechst 33258 at room temperature in the dark for 10 min. Samples were observed under a fluorescent microscope (Nikon UFX-II, Japan).

2.7. Analysis of apoptosis rate by flow cytometry

To quantify the apoptotic cells, PC12 cells were harvested after exposed to 200 μM H_2O_2 for 12 h and washed twice with cold PBS and fixed with 70% ethanol at 4 °C for 24 h. The cells were then centrifuged, and ethanol was removed by washing thoroughly with PBS. The cells were resuspended in 100 μl PBS containing 200 μg/ml

Table 1
Protective effect of hopeahainol A against H_2O_2-induced PC12 cell injury.

Treatment	MTT reduction (%)	LDH release (U/l)
Control	100 ± 4.9	98.1 ± 4.6
H_2O_2	65.5 ± 6.0[#]	171.0 ± 10.6[#]
Hopeahainol A (0.1 μM) + H_2O_2	72.2 ± 6.7	143.1 ± 5.6[**]
Hopeahainol A (1 μM) + H_2O_2	74.8 ± 5.8[*]	122.4 ± 9.8[**]
Hopeahainol A (10 μM) + H_2O_2	80.9 ± 8.5[**]	111.1 ± 9.2[**]

Cells were incubated with 200 μM H_2O_2 for 6 h for MTT and LDH assay. Hopeahainol A was added to the culture 30 min prior to H_2O_2 addition. The data presented as mean ± S.D. ($n = 6$). A difference was considered statistically significant when [*]$p < 0.05$ and [**]$p < 0.01$ vs. H_2O_2 group or [##]$p < 0.01$ vs. control.

propidium iodide and 50 μg/ml RNase A at room temperature for 30 min and measured with a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). The percentage of apoptotic cell accumulation in the sub-G1 peak was calculated.

2.8. Analysis of ROS

The cellular ROS was quantified using DCFH-DA according to Hong and Joseph (1999). The cells on black 96-well plates were incubated with 100 μM DCFH-DA in the loading medium in 5% CO_2/95% air at 37 °C for 30 min. After DCFH-DA was removed, the cells were washed once with phenol red free RPMI 1640 medium and then incubated in RPMI 1640 medium containing different concentrations of hopeahainol A (0.1–10.0 μM) for 30 min, followed by the addition of 200 μM H_2O_2. After 30 min incubation in 5%CO_2/95% air at 37 °C, DCFH-DA-loaded cells were read in a Safire (Tecan, Crailsheim, Germany) fluorescence plate reader (excitation, 485 ± 12 nm; emission, 530 ± 12 nm). The percentage increase in fluorescence per well was calculated by the formula $[(Ft_{30} - Ft_0)/Ft_0 \times 100]$, where Ft_0 is the fluorescence at time 0 min and Ft_{30} is the fluorescence at time 30 min in the presence of H_2O_2.

2.9. Measurement of intracellular Ca^{2+} ($[Ca^{2+}]_i$)

$[Ca^{2+}]_i$ was monitored using the fluorescent Ca^{2+}-sensitive dye, Fura 2-acetoxymethy ester (Fura 2-AM) (Liu et al., 2007). The confluent monolayer of PC12 cells was preloaded with Fura 2-AM (5 μM) for 30 min at room temperature in the dark at 37 °C in a humidified incubator. After loading with Fura 2-AM, cells were collected and gently rinsed three times with D-Hanks' solution, then resuspended in D-Hanks' solution containing 0.2% BSA at 10^6 cell/ml. The Fura 2-AM loaded cells were incubated with different concentrations of hopeahainol A (0.1–10.0 μM) at 37 °C for 30 min. Then 200 μM H_2O_2 was added and incubated for another 30 min. The fluorescence was measured at emission wavelength of 510 nm and excitation wavelengths of 340 and 380 nm on a Safire (Tecan, Crailsheim, Germany) fluorescence plate reader. The fluorescence ratio (F340/F380) was calculated as an indicator of $[Ca^{2+}]_i$.

2.10. Measurement of MMP

MMP was monitored using the fluorescent dye Rhodamine 123, a cell permeable cationic dye. Rhodamine 123 was added to cell cultures to attain a final concentration of 10 μM for 30 min at 37 °C after cells were treated with H_2O_2 for 6 h. The cells were collected and washed twice with PBS and then analyzed by FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) (Shimizu et al., 1996).

2.11. Caspase-3, -8 and -9 activity assay

Caspases activities were measured using caspase activity assay kit (Beyotime, C1115, C1151 and C1157) according to the manufacturer's instructions. Briefly, cultured PC12 cells (($3–5) \times 10^6$) were washed with cold PBS twice, resuspended in lysis buffer and left on ice for 20 min. The lysate was centrifuged at $16,000 \times g$ at 4 °C for 3 min. Caspase-3, -8 and -9 activities were measured by using reaction buffer (containing DTT) and caspases substrate peptides Ac-DEVD-pNA, Ac-IETD-pNA and Ac-LEHD-pNA, respectively. The release of p-nitroanilide (pNA) was qualified by determining the absorbance with Tecan SUNRISE at 405 nm. The fold increase in absorbance was calculated by the formula $[OD_i/OD_0]$, where OD_0 is the absorbance without the H_2O_2 injury (control) and OD_i is the absorbance with the H_2O_2 injury and hopeahainol A treatment at the indicated concentration.

2.12. Statistical analysis

Data were expressed as mean ± standard deviations (S.D.) and analyzed by Student's t-test. A p-value of 0.05 or less was considered as statistically significant.

Table 2
Effects of hopeahainol A on lipid peroxidation and antioxidant enzyme activities in H_2O_2-treated PC12 cells.

Treatment	MDA (nmol/mg protein)	CAT (U/mg protein)	GGSH-Px (U/mg protein)
Control	0.39 ± 0.04	11.0 ± 1.1	14.7 ± 0.8
H_2O_2	1.12 ± 0.06[#]	2.5 ± 0.7[#]	2.5 ± 0.3[#]
Hopeahainol A (0.1 μM) + H_2O_2	0.55 ± 0.05[**]	3.6 ± 0.4[*]	4.9 ± 0.4[**]
Hopeahainol A (1 μM) + H_2O_2	0.47 ± 0.06[**]	3.9 ± 0.5[**]	7.2 ± 0.9[**]
Hopeahainol A (10 μM) + H_2O_2	0.43 ± 0.05[**]	5.1 ± 0.7[**]	11.5 ± 1.0[**]

Cells were incubated with 200 μM H_2O_2 for 6 h for assay of MDA, SOD, CAT and GSH-Px. Hopeahainol A was added to the culture 30 min prior to H_2O_2 addition. The data presented as mean ± S.D. (n = 6). A difference was considered statistically significant when [*] $p < 0.05$ and [**] $p < 0.01$ vs. H_2O_2 group or [##] $p < 0.01$ vs. control.

3. Results

3.1. Hopeahainol A protected PC12 cells against H_2O_2-induced cytotoxicity

As estimated by MTT assay, cell viability was markedly decreased after a 6-h exposure to 200 μM H_2O_2. However, when cells were pre-incubated with hopeahainol A (0.1–10.0 μM) for 30 min, cell toxicity was significantly attenuated in a dose-dependent manner (Table 1). Hopeahainol A at concentration of 1 μM rescued the H_2O_2-induced decrease in viability rate by about 75%. To further investigate the protective effect of hopeahainol A, the release of LDH, another indicator of cell toxicity, was performed. As shown in Table 1, a significant increase in LDH release was observed after 6-h exposure to 200 μM H_2O_2. Hopeahainol A attenuated markedly this increase. The protective effect of hopeahainol A on H_2O_2-induced cytotoxicity determined by LDH assay was similar to that determined by MTT assay.

3.2. Hopeahainol A reduced lipid peroxidation and rescued loss of antioxidant enzyme activities in H_2O_2-treated PC12 cells

Treatment of PC12 cells with 200 μM H_2O_2 caused the increase of the intracellular MDA level, while pre-incubation of cells with 1 and 10 μM hopeahainol A markedly attenuated the increase (Table 2). In addition, treatment of PC12 cells with 200 μM H_2O_2 caused the decrease in the activities of CAT and GSH-Px, respectively. Pretreatment with hopeahainol A significantly attenuated the changes of CAT and GSH-Px activities in a dose-dependent manner (Table 2).

3.3. Hopeahainol A protected PC12 cells against H_2O_2-induced apoptosis

Apoptotic cells were visualized by using the DNA-binding fluorescent dye Hoechst 33258. After treatment by H_2O_2 at a concentration of 200 μM for 12 h, cell nuclei became increasingly bright. Finally, it decreased in size and fragmented into apoptotic bodies. In contrast, pre-incubated with hopeahainol A for 30 min, cells appeared remarkably preserved and those alterations were significantly attenuated (Fig. 2). The apoptosis rate was defined as the percentage of cells with subdiploid DNA content (DNA fragmentation) determined by flow cytometry. The sub-G1 peak in flow cytometry detection was considered as an indicator of cell apoptosis. A significant increase in the apoptosis rate (from 1.68% to 11.58%) was found when cells were treated with 200 μM H_2O_2 for 12 h. However, when cells were pretreated with 0.1, 1 and 10 μM hopeahainol A for 30 min before the addition of H_2O_2, the percentage of apoptosis was decreased from 11.58% to 10.93%, 8.52% and 4.86%, respectively (Fig. 3).

3.4. Hopeahainol A inhibited the H_2O_2-induced intracellular accumulation of ROS

To determine whether hopeahainol A could prevent H_2O_2-induced ROS generation and the resulting oxidative stress, levels of
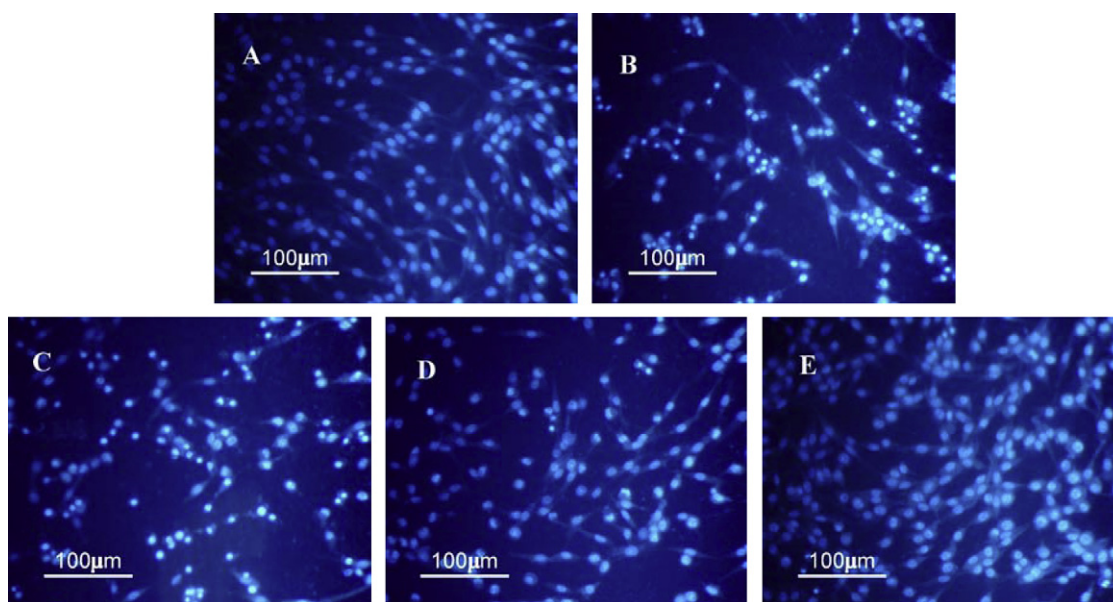

Fig. 2. Morphological analysis of nuclear chromatin by Hoechst 33258. PC12 cells were treated with H_2O_2 in the absence or in the presence of hopeahainol A. (A) Control conditions. (B) After exposure to 200 μM H_2O_2 for 12 h, cells displayed condensed chromatin and apoptotic nuclei. (C–E) PC12 cells were pretreated with 0.1, 1 or 10 μM hopeahainol A of hopeahainol A for 30 min before exposure to 200 μM H_2O_2 for 12 h.
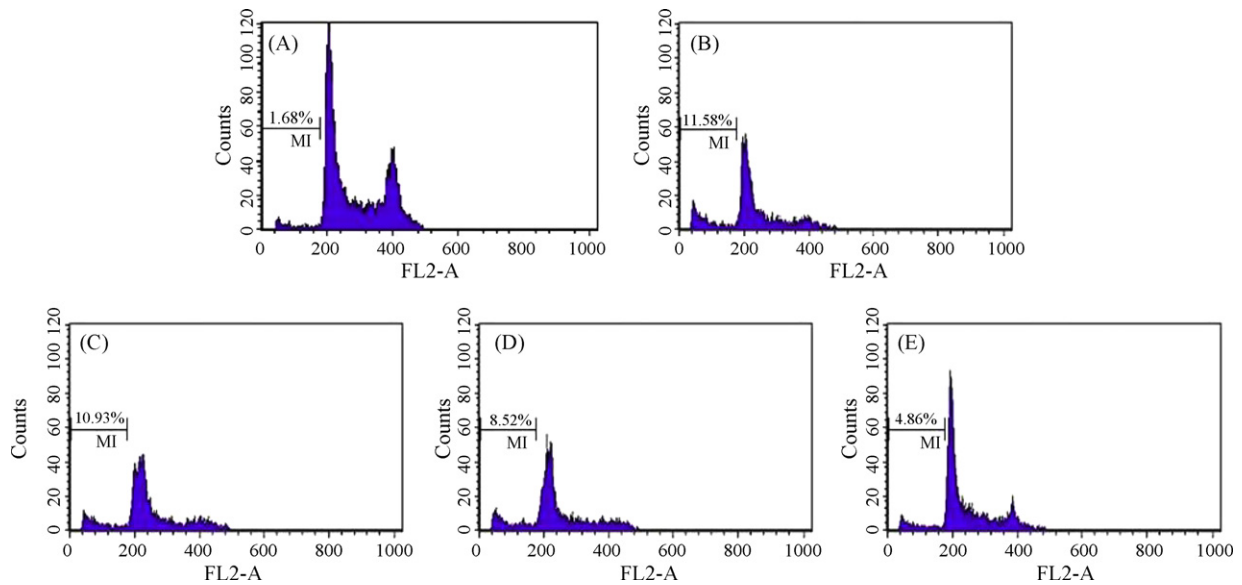
Fig. 3. Inhibitory effect of hopeahainol A on H_2O_2-induced apoptosis in PC12 cells as determined by flow cytometric assay. (A) Control; (B) PC12 cells exposed to 200 μM H_2O_2 for 12 h; (C–E) PC12 cells pretreated with 0.1 μM, 1 μM or 10 μM hopeahainol A for 30 min followed by exposure to 200 μM H_2O_2 for additional 12 h.

ROS production in the cells were determined using the fluorescence probe DCFH$_2$-DA. PC12 cells exposed to H_2O_2 for 30 min displayed a significant increase in the intracellular level of ROS relative to the levels seen in control cells. PC12 cells pretreated with 1.0 and 10 μM hopeahainol A before H_2O_2 exposure markedly reduced the ROS levels in PC12 cells. However, 0.1 μM hopeahainol A has no significant effect on the ROS levels of the H_2O_2-treated PC12 cells (Fig. 4).

3.5. Hopeahainol A inhibited H_2O_2-induced increase of $[Ca^{2+}]_i$ in PC12 cells

As shown in Fig. 5, treatment of PC12 cells with H_2O_2 for 30 min increased the fluorescence ratio (F340/F380) to 161.6% compared to untreated control cultures. When the cells pretreated with 10 μM hopeahainol A, F340/F380 was reduced from 161.6% to 121.3% significantly.
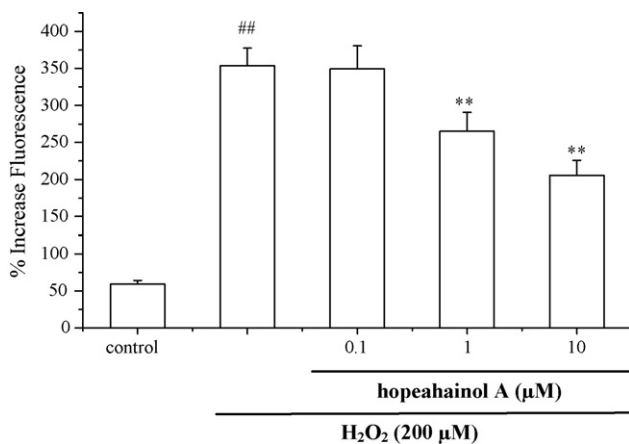

Fig. 4. Effect of hopeahainol A on H_2O_2-induced intracellular accumulation of ROS. Intracellar ROS levels were determined based on the peroxide-sensitive DCFH$_2$-DA fluorescence in Section 2. The data, expressed as % increase fluorescence, are mean ± S.D. ($n = 6$). A difference was considered statistically significant when [**]$p < 0.01$ vs. H_2O_2 group or [##]$p < 0.01$ vs. control.

3.6. Hopeahainol A prevented loss of MMP in PC12 cells

To assess the effect of hopeahainol A on the changes in MMP induced by H_2O_2 in PC12 cells, flow cytometric analyses were carried out using Rhodamine 123. After incubation of PC12 cells with 200 μM H_2O_2 for 6 h, the MMP was detected by the weakening of the fluorescence intensity of Rhodamine 123. As compared to control cells, H_2O_2 treatment increased the Rhodamine 123 negative cells from 9.45% to 31.77%. Pretreatment with 0.1, 1 and 10 μM of hopeahainol A protected cells against the H_2O_2-induced lowering of MMP, decreasing Rhodamine 123 negative cells to 24.60%, 23.65% and 18.12%, respectively (Fig. 6).

3.7. Hopeahainol A inhibited H_2O_2-induced increases of Caspase-3, -8 and -9 activities in PC12 cells

The apoptotic process included the activation of cysteine proteases, which represent both initiators and executors of cell death. Fig. 7 showed that H_2O_2 treatment caused remarkable increases of caspase-3, -8 and -9 proteolytic activities in PC12 cells. Pretreatment with 0.1, 1 and 10 μM of hopeahainol A protected cells against the H_2O_2-induced increases of caspase-3, -8 and -9 activities significantly.

4. Discussion

Hopeahainol A, a natural phenolic compound, was reported to be AChE-inhibitory (Ge et al., 2008). Considering the polyphenolic structure of hopeahainol A, we assumed that hopeahainol A may have the neuroprotective effects via reducing oxidative stress. To investigate the neuroprotective effect by hopeahainol A in oxidative stress-induced neuronal toxicity, the H_2O_2-treated PC12 cell culture system, an usual in vitro model for neuronal injury test (Silva et al., 2006; Mehrani and Golmanesh, 2008; Grant and Griffiths, 2007; Huang et al., 2008), was used in this study.

ROS readily damage biological molecules and ultimately lead to apoptotic or necrotic cell death (Fiers et al., 1999). In this study, treatment of PC12 cells with 200 μM H_2O_2 for 6 h caused a marked decrease in cell survival and elevation of oxidative stress characterized by MDA production and LDH release. Hopeahainol A remarkably reduced the cell death, MDA production and LDH
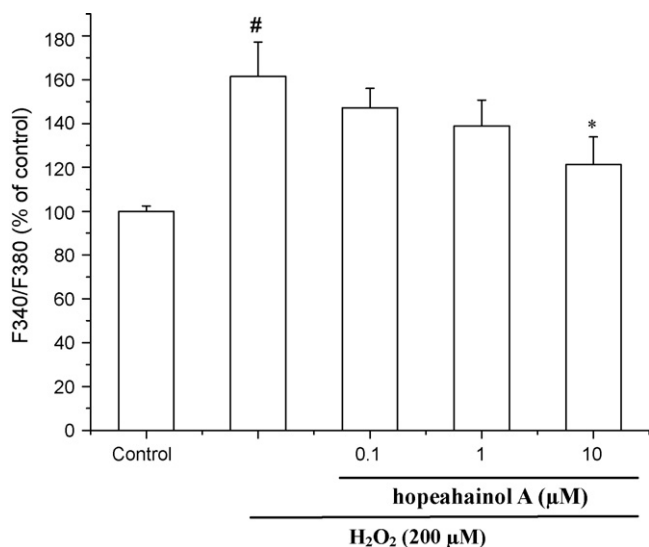
Fig. 5. Effects of hopeahainol A on the increase of $[Ca^{2+}]_i$ induced by H_2O_2 in PC12 cells. After loading with Fura 2-AM, the cells were incubated with hopeahainol A for 30 min at 37 °C. Then 200 μM H_2O_2 was added for 30 min and $[Ca^{2+}]_i$ was measured by fluorescence analysis. The data were presented as mean ± S.D. ($n=4$). A difference was considered statistically significant when $^*p < 0.05$ vs. H_2O_2 group or $^\#p < 0.05$ vs. control.

Fig. 7. Effects of hopeahainol A on H_2O_2-induced increases of caspase activities. The data were presented as mean ± S.D. ($n=4$). A difference was considered statistically significant when $^*p < 0.05$ and $^{**}p < 0.01$ vs. H_2O_2 group or $^{\#\#}p < 0.01$ vs. control.

release. In addition, exposed to 200 μM H_2O_2 for more than 12 h, PC12 cells showed typical characteristics of apoptosis, such as chromosome condensation and internucleosomal DNA fragmentation. Hopeahainol A could reduce the apoptotic morphological changes and the sub-G1 DNA fraction in H_2O_2-treated PC12 cells. All these suggested that hopeahainol A possessed protective effect against H_2O_2-induced injury in PC12 cells.

One possible mechanism of neuroprotective effects of hopeahainol A might be related to the modulation of endogenous antioxidant enzymes. H_2O_2 can generate the more detrimental hydroxyl radical and increase the ROS levels in cells. Excessive ROS can lead to lipid peroxidation, oxidation of proteins, and DNA
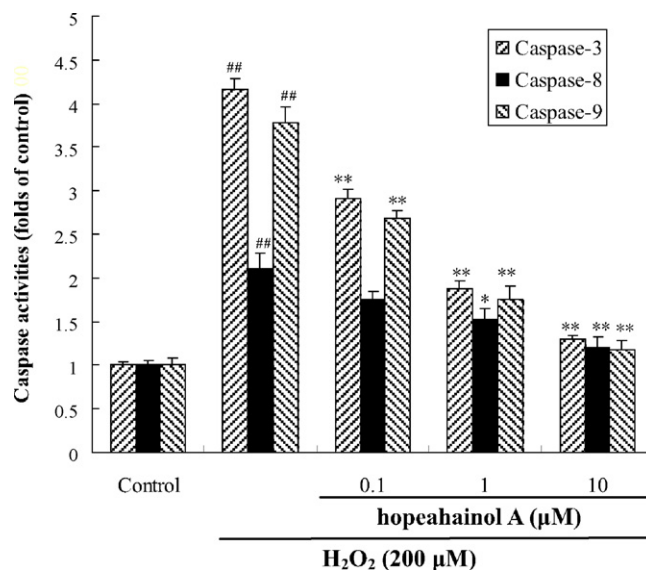
damage (Götz et al., 1994). The H_2O_2 in the cells is removed by CAT, which is active only against H_2O_2 and by GSH-Px, which can also act on lipid hydroperoxides (Chance et al., 1979). The combined action of these two enzymes provides a repair mechanism for oxidized membrane components. It was reported that GSH-Px and catalase activities in PC12 cells were reduced after the treatment of H_2O_2 (Wu et al., 2006). In this study, H_2O_2-induced decrease in cell survival was also correlated with reduction in ACT and GSH-Px activities. When PC12 cells were pre-incubated with hopeahainol A, an elevation in activities of CAT and GSH-Px as well as cell survival were observed. It suggested that the modulation of endogenous antioxidant enzymes was involved in cytoprotective effects of hopeahainol A. The mechanism of hopeahainol A on
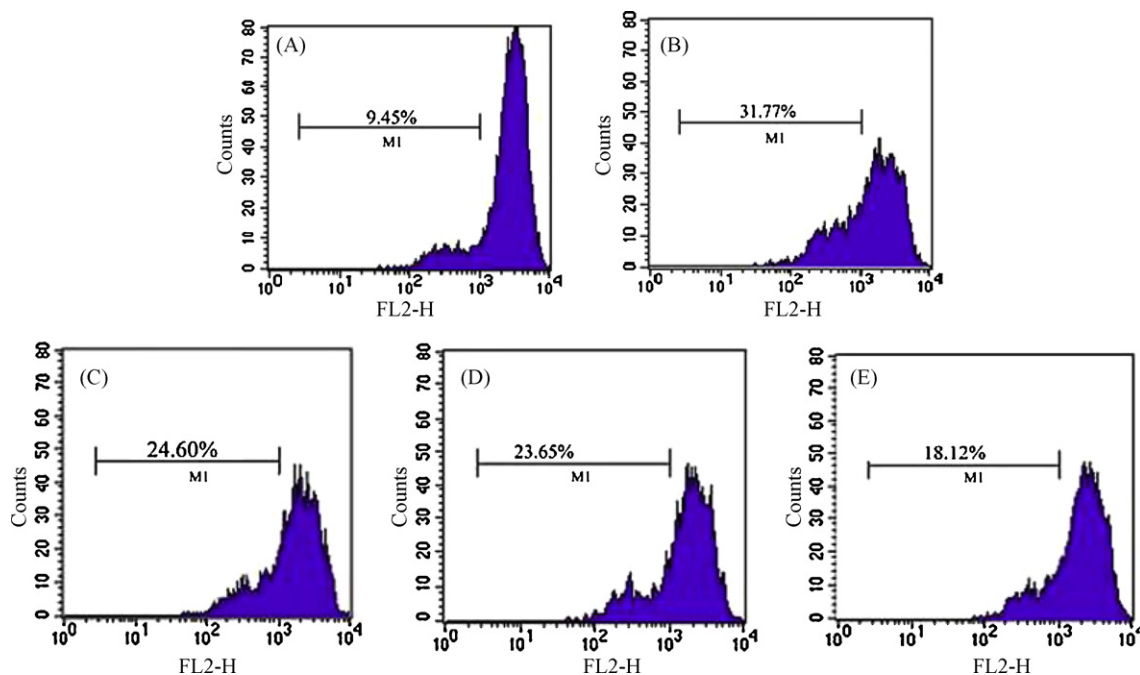

Fig. 6. Effects of hopeahainol A on H_2O_2-induced reduction of mitochondrial membrane potential. (A) control; (B) PC12 cells exposed to 200 μM H_2O_2 for 12 h; (C–E) PC12 cells pretreated with 0.1, 1 or 10 μM hopeahainol A for 30 min followed by exposure to 200 μM H_2O_2 for additional 12 h.

antioxidant enzyme activities in H_2O_2-treated PC12 cells remained unclear.

Another possible mechanism of neuroprotective effects of hopeahainol A might be its direct scavenging of ROS produced by H_2O_2, since polyphenols can scavenge different kinds of ROS and organic free radicals such as superoxide anion, hydroxyl radicals, and lipid free radicals (Frade et al., 2005; Ishige et al., 2001). As one of the main ROS, H_2O_2 can elevate oxidative stress, resulting in rising Ca^{2+} concentration in the cytoplasm (Wang and Joseph, 2000). Marked elevation in Ca^{2+} then causes the new ROS formation (Tan et al., 1998). On the other hand, the increase of Ca^{2+} and ROS may impair mitochondrial function (Trump and Berezesky, 1995), leading to a significant decrease in MMP. Disruption of MMP causes the release of apoptosis-inducing factors which activate caspase cascade, cause nuclear condensation, and generate secondary ROS (Petit et al., 1996), and as a result, leads to apoptosis. In this study, treatment of PC12 cells with H_2O_2 caused the intracellular accumulation of ROS and Ca^{2+} and further induced the loss of MMP. And then the disruption of MMP caused the increase of caspase-3, -8 and -9 activities and eventually the apoptosis. However when PC12 cells were pretreated with hopeahainol A, the accumulation of ROS and Ca^{2+}, the loss of MMP, the increase of caspase-3, -8 and -9 activities and apoptosis were attenuated in a dose-dependent manner. These results suggested that ROS scavenging effects of hopeahainol A might be an important factor in reducing the level of the cell death and apoptosis induced by H_2O_2.

It was reported that the $\alpha7$ nicotinic acetylcholine receptors mediated the neuroprotective effects of AChEIs (Arias et al., 2005). AChEIs increased availability of acetylcholine by inhibiting AChE and by subsequently activating $\alpha7$ nicotinic acetylcholine receptors (Ezoulin et al., 2008). So, another possible mechanism of neuroprotective effects of hopeahainol A might be associated with its AChE-inhibition activity since several AChEIs possessed the similar effects on the H_2O_2-treated PC12 cells (Zhang and Tang, 2000; Xiao et al., 2000).

In summary, the results of the current study suggested that hopeahainol A was able to protect cultured PC12 cells against damage induced by H_2O_2. These effects of hopeahainol A were, at least in part, attributed to its AChE-inhibition activity, antioxidant property and ability of modulating endogenous antioxidant enzymes. These data, along with the acetylcholinesterase-inhibition activity of hopeahainol A, suggested that there was potential for hopeahainol A to be used in treating AD that involved acetylcholinesterase, free radical and oxidative damage. Furthermore the neuroprotective activity discerned with hopeahainol A demonstrated that the polyphenol is an antioxidative AChEI highly desired for the treatment of Alzheimer's disease.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgements

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