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Protective effect of protocatechuic acid from *Alpinia oxyphylla* on hydrogen peroxide-induced oxidative PC12 cell death

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Abstract

The neuroprotective effects of protocatechuic acid (PCA), a phenolic compound isolated from the kernels of *Alpinia oxyphylla*, on hydrogen peroxide (H_2O_2)-induced apoptosis and oxidative stress in cultured PC12 cells were investigated. Exposure of PC12 cells to 0.4 mM H_2O_2 induced a leakage of lactate dehydrogenase (LDH) and decreased cell viability denoted by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. PCA increased PC12 cellular viability and markedly attenuated H_2O_2-induced apoptotic cell death in a dose-dependent manner. By flow cytometric analysis, PCA showed its significant effect on protecting PC12 cells against H_2O_2-induced apoptosis. In these cells, the levels of glutathione (GSH) and activity of catalase were augmented, while glutathione peroxidase activity remained unchanged. In addition, PCA also protected against cell damage induced by H_2O_2 and Fe^{2+}, which generated hydroxyl radicals (OH) by the Fenton reaction. These results suggest that PCA may be a candidate chemical for the treatment of oxidative stress-induced neurodegenerative disease.

Keywords: Protocatechuic acid; Hydrogen peroxide; Oxidative stress; Apoptosis; PC12 cells

1. Introduction

Oxidative stress has been implicated as a major cause of cellular injures in a vast variety of clinical abnormalities including neurodegenerative disorders (Olanow, 1993; Markesbery, 1997; Halliwell and Gutteridge, 1999a). It is mediated by reactive oxygen species, including free radicals such as superoxide ions (O_2^-) and hydroxyl radicals (OH) as well as non-free radical species such as hydrogen peroxide (H_2O_2) (Halliwell and Gutteridge, 1999b), which are generated as byproducts of normal and aberrant metabolic processes that utilize molecular oxygen. Reactive oxygen species cause oxidative damage to various biological macromolecules including DNA, lipids, and proteins, thereby disrupting cellular function and integrity (Gardner et al., 1997; Fiers et al., 1999). Studies have shown that biological systems have evolved with endogenous defense mechanisms to help protect against reactive oxygen species-induced cell damage. Superoxide dismutase, catalase, and glutathione peroxidase are endogenous antioxidant enzymes, which play pivotal roles in preventing cellular damage caused by reactive oxygen species. They require micronutrient as cofactors such as selenium, iron, copper, zinc, and manganese for optimum catalytic activity and effective antioxidant defense mechanisms (Halliwell and Gutteridge, 1992; Halliwell, 2001). A low steady-state level of intracellular superoxide is maintained by superoxide dismutase, and H_2O_2, generated by superoxide dismutase is removed by catalase or glutathione peroxidase, which also acts on lipid hydroperoxides. It is well known that many types of chemical and physiological inducers of oxidative stress are able to cause apoptotic cell death (Slater et al., 1995; O'Brien et al., 2000). For instance, H_2O_2 is one of representative reactive oxygen species that is produced during the redox process and is recently considered to play a role as a messenger in intracellular signaling cascades (Finkel, 1998; Rhee, 1999). Under biological conditions where free iron is released from the storage pool, the toxicity of H_2O_2, is partly attributed to (OH) generated by a Fenton-type reaction (Stadtman and Berlett, 1991).

There have been considerable efforts to search for natural substances for the neuroprotective potential, attention has been focused on a wide array of dietary antioxidants that can scavenge free radicals and protect cells from oxidative damage, such as resveratrol (Jang and Surh, 2001). *Alpinia* (*A.*) *oxyphylla* MIQUEL (Zingiberaceae) is an important traditional Chinese medicinal herb whose fruits are widely used as a tonic, aphrodisiac,

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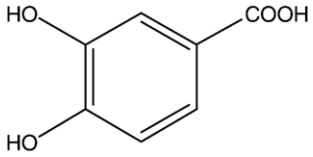

Fig. 1. The chemical structure of protocatechuic acid (PCA).

anti-salivation, anti-polyuria, and anti-diarrhea according to the Chinese Pharmacopoeia. There have been growing evidences that the extract from the kernels of *A. oxyphylla* possesses significant neuroprotective activity (Xinyu et al., 2003; Koo et al., 2004; Wong et al., 2004), although little is yet known about the pharmacological effects or active ingredients. In our search for new active ingredients from traditional Chinese medicinal herbs, protocatechuic acid (PCA, Fig. 1), a simple phenolic compound, was isolated from the kernels of *A. oxyphylla* through bioassay-guided fractionation (An et al., 2006). Recently, PCA has been demonstrated to show strong antioxidant and antitumor promotion effects (Tseng et al., 1996, 1998) and induced apoptosis in HL-60 human leukemia cells (Tseng et al., 2000). In contrast to the antioxidative effects of PCA demonstrated in nonneural cells, however, very little research has been done on the neuroprotective properties, especially at the cellular level. Therefore, in the present study, we examined the protective effects of PCA on H_2O_2-induced cytotoxicity in cultured PC12 cells that retain dopaminergic characteristics and have been widely used for neurobiological and neurochemical studies.

2. Materials and methods

2.1. Materials

PCA was isolated from *A. oxyphylla*, and its structure has been described previously (An et al., 2006). The purity of the compound was more than 98% by high-performance liquid chromatography (HPLC) analysis. The reagents used enzyme assays were obtained from Jiancheng Bioengineering Institute (Nanjing, China). The ApoGSH™ Glutathione Colorimetric Detection Kit was from BioVision Inc. (CA, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), propidium iodide, and other chemicals were purchased from Sigma-Aldrich Inc. (St. Louis, Mo, USA).

2.2. Cell culture

Rat PC12 cells (adrenal gland; pheochromocytoma) were obtained from Institute of Biochemistry and Cell Biology, SIBS, CAS. PC12 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated horse serum, 5% fetal bovine serum (FBS) (Gibco, MD, USA), 100 U/ml penicillin and 100 μg/ml streptomycin in a water-saturated atmosphere of 5% CO_2 at 37 °C. The culture medium was changed every three days and cells were subcultured about once a week. PC12 cells were differentiated with 100 ng/ml nerve growth factor-7S (Gibco, MD, USA), which was added to the culture medium with incubation continued for 9 days (Tatton et al., 2002). Cells were washed with DMEM medium containing 1% FBS 24 h before experiments and replated in the 96 and 24 well plates.

2.3. Analysis of cell viability

Cell viability was evaluated by the reduction of MTT (Hansen et al., 1989; Vian et al., 1995). Briefly, PC12 cells (1×10^5 cells/ml) were treated with 0.4 mM H_2O_2 in the presence of PCA for 24 h at 37 °C. After 3 h incubation with MTT (0.5 mg/ml), cells were lysed in dimethyl sulfoxide (DMSO) and the amount of MTT formazan was qualified by determining the absorbance at 570 and 630 nm using a microplate reader (SPECTRAFLUOR, TECAN, Sunrise, Austria). Cell viability was expressed as a percent of the control culture value.

2.4. Detection of apoptosis

PC12 cells (1×10^5 cells/ml) were treated with 0.4 mM H_2O_2 in the presence of PCA for 24 h at 37 °C, and then analyzed by flow cytometry assays. Cells were harvested by centrifugation at 1000 ×*g* for 5 min and washed with ice-cold PBS and fixed with 70% cold ethanol at 4 C for 1 h. The fixed cells were washed with PBS and resuspended in a staining solution containing propidium iodide (PI) (20 μg/ml) and Dnase-free RNase (100 μg/ml). The cell suspension were incubated at 37 °C for 30 min in the dark and quantitatively analyzed by a FACScan flow cytometer (Beckman Coulter, Epics) equipped with the SYSTEM II™ analysis software.

2.5. Measurement of total glutathione

PC12 cells (1×10^5 cells/ml) were treated with 0.4 mM H_2O_2 in the presence of PCA for 24 h at 37 °C, and the total glutathione (reduced form GSH+oxidized form GSSG) was determined according to the user's manual for the ApoGSH™ Glutathione Colorimetric Detection Kit (BioVision). Briefly, cells (5×10^5 cells) were centrifuged at 700 ×*g* for 5 min at 4 °C and the supernatants removed. The pellets were washed with ice-cold PBS, lysed in 80 μl ice-cold Glutathione Buffer and incubated on ice for 10 min. Then the samples were dissolved with 5% 5-sulfosalicylic acid (SSA, 20 μl) and centrifuged at 8000 ×*g* for 10 min. The supernatants (20 μl) were incubated in 160 μl of the Reaction Mix at room temperature for 10 min and Substrate solution (20 μl) was added and the mixture was incubated for a further 10 min. Absorbance was measured at 415 nm using a microplate reader. The standard curve was obtained from absorbance of the diluted GSH Standard that was incubated in the mixture as in samples.

2.6. Assays for lactate dehydrogenase (LDH), catalase, and glutathione peroxidase

PC12 cells were washed with ice-cold PBS, harvested by centrifugation at 1000 ×*g* for 5 min, pooled in 0.5 ml of 0.1 M phosphate buffer (pH 7.4) and homogenized. The homogenate was centrifuged at 3000 ×*g* for 20 min at 4 °C, and the supernatant was used for the activity assay according to the manufacturer instructions. LDH leakage was calculated as the

percentage of LDH in the medium versus total LDH activity in the cells. Activities of catalase and glutathione peroxidase were expressed relative to the amount of protein in the cell extracts determined by the method of Bradford (1976).

2.7. Statistical analysis

Data were expressed as the mean±S.E.M. from three independence experiments and evaluated using one-way ANOVA followed by Student's *t*-test. Significant differences were established at $P<0.05$.

3. Results

3.1. Effects of PCA from A. oxyphylla on cell viability

PC12 cells were treated with different amounts of PCA from *A. oxyphylla* (0.006 to 1.2 mM) for 24 h. The effect of PCA on cell viability was evaluated by MTT assay. As shown in Fig. 2, at concentrations from 0.3 to 1.2 mM, PCA slightly increased cell viability and did not induce changes in cell morphology. The treatment with 1.2 mM concentration of PCA increased the MTT reduction activity by 113.1% of control group.

On the other hand, the effect of PCA on the H_2O_2-induced cell damage was assessed. A dose- and time-dependent increase in LDH leakage was demonstrated in PC12 cells exposed to H_2O_2. At a concentration greater than 0.2 mM, LDH leakage was significantly increased compared with that of untreated cells. Furthermore, MTT assay also showed a dose-dependent decrease in cell viability. Significant reduction in viability was observed in the cells treated with greater than 0.4 mM H_2O_2 (data not shown). Therefore, it was decided to use a concentration of 0.4 mM for the subsequent experiments. The results in Fig. 3A showed that treatment of PC12 cells with PCA dose-dependently suppressed H_2O_2-induced leakage of LDH, and these findings were further verified by MTT assay (Fig. 3B). Thus, it was possibly concluded that PCA is effective for the protection and viability of PC12 cells.

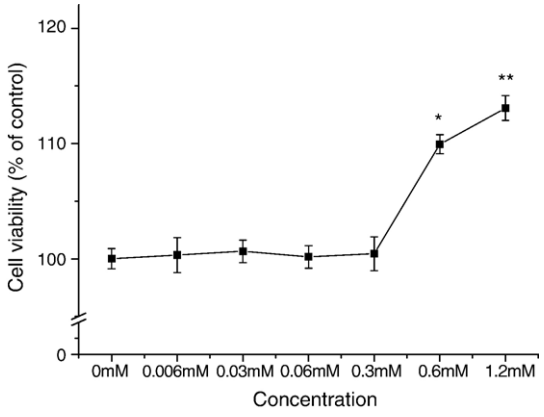

Fig. 2. Effects of PCA from *A. oxyphylla* on cultured PC12 cell viability. Cells (1×10^5 cells/ml) were treated with PCA for 24 h at 37 °C. Cell viability was assessed by the MTT method as described in Materials and methods. Data are mean±S.E.M. values obtained from four culture wells per experiment, determined in three independent experiments. (*) $P<0.05$, and (**) $P<0.01$, compared with the control group.

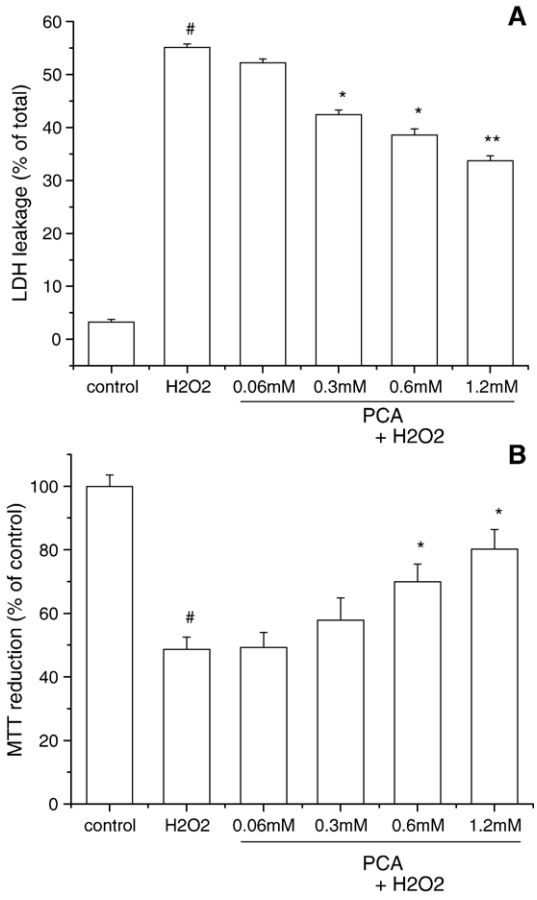

Fig. 3. Effects of PCA from *A. oxyphylla* on H_2O_2-induced cell damage. PC12 cells (1×10^5 cells/ml) were treated with 0.4 mM H_2O_2 in the absence or presence of PCA. LDH leakage (A) and viability of the cells (B) were determined after 24 h. Data are expressed as percent of values in untreated control cultures, and are means±S.E.M. of four experiments. ($^{\#}$) $P<0.05$ in comparison with control, (*) $P<0.05$ and (**) $P<0.01$ in comparison with cells exposed to H_2O_2 alone.

3.2. Effects of PCA from A. oxyphylla on H_2O_2-induced apoptotic cell death

In order to analyze the protective effect of PCA on H_2O_2-induced cell injury, we investigated the effect on the nuclear changes by flow cytometry. Flow cytometry detects apoptotic dead cells as well as those with fragmented nuclei, which are also called sub-G1 cells. As shown in Fig. 4, analysis of DNA contents following 0.4 mM H_2O_2 treatment of PC12 cells revealed an increase in the proportion of cells with sub-G1 DNA content to 11.3% (Fig. 4C). Treatment with 0.4 mM H_2O_2 in the presence of 1.2 mM PCA reduced the apoptotic sub-G1 population to 8.3% (Fig. 4D).

3.3. Effects of PCA from A. oxyphylla on cellular total glutathione levels and on the activities of catalase and glutathione peroxidase in H_2O_2-induced PC12 cells

Studies have shown that cell is well equipped with defense mechanisms against oxidative stress-induced cell damage (Halliwell and Gutteridge, 1999a). Glutathione (GSH) is the major intracellular low-molecular-weight thiol that plays a critical role

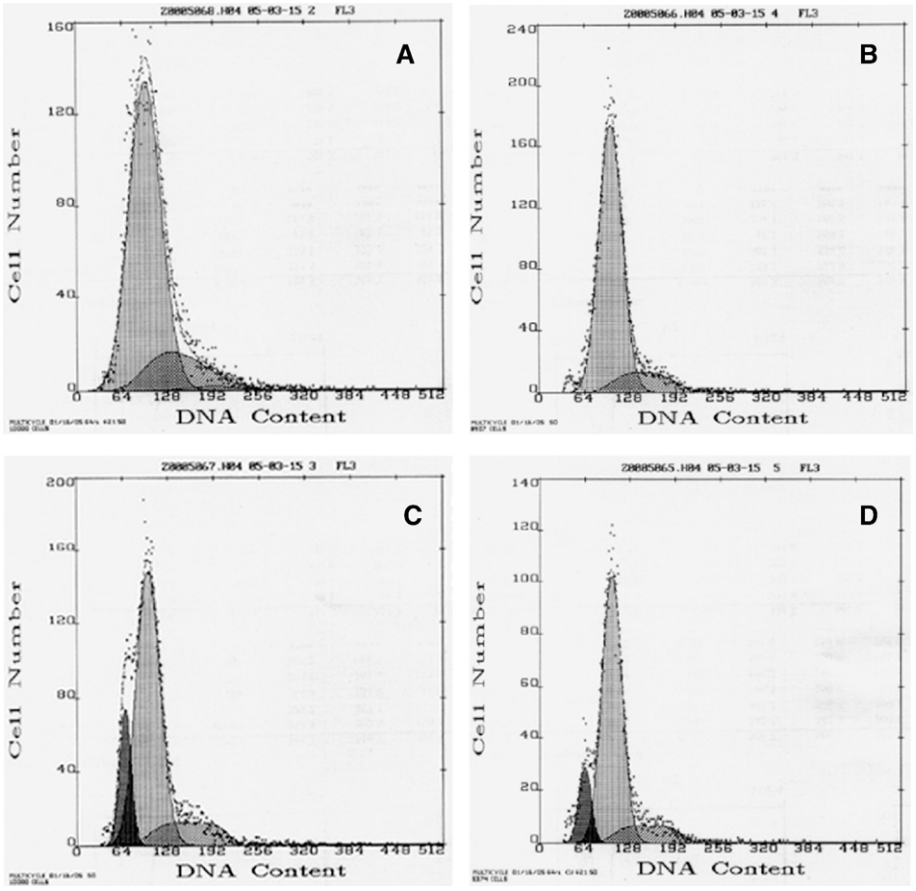

Fig. 4. Effects of PCA from *A. oxyphylla* against H_2O_2-induced apoptosis in cultured PC12 cells by flow cytometric DNA analysis. PC12 cells (1×10^5 cells/ml) were treated with 0.4 mM H_2O_2 in the presence of 1.2 mM PCA for 24 h at 37 °C. (A) control cells; (B) cells treated with PCA alone; (C) cells treated with H_2O_2 alone; (D) cells treated with H_2O_2 and PCA.

in the cellular defense against oxidative stress in mammalian cells. So we assessed the effect of PCA on the level of cellular GSH. As shown in Fig. 5, the GSH content was significantly depleted in the cells exposed to H_2O_2. Although treatment of the cells with PCA alone did not affect the cellular total GSH levels, it was effective to attenuate the reduction of GSH levels in H_2O_2-induced PC12 cells.

H_2O_2 is decomposed by catalase or glutathione peroxidase. We therefore examined the effect of PCA on catalase and glutathione peroxidase activities in PC12 cells. As shown in

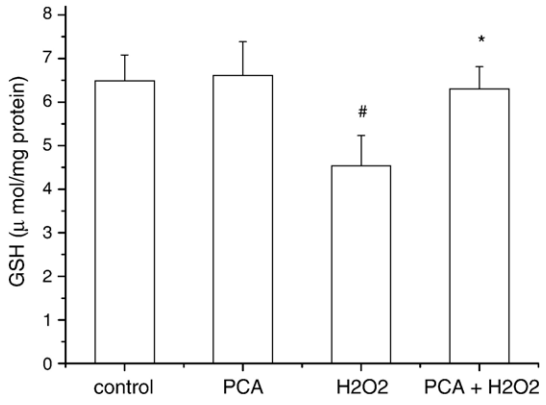

Fig. 5. Effects of PCA from *A. oxyphylla* on cellular total GSH levels in H_2O_2-induced PC12 cells. Cells (1×10^5 cells/ml) were treated with 0.4 mM H_2O_2 in the absence or presence of 1.2 mM PCA for 24 h at 37 °C. Data are mean ± S.E.M. values obtained from three culture wells per experiment, determined in three independent experiments. (#) $P<0.05$ in comparison with control, and (*) $P<0.05$ statistically different from H_2O_2-induced group.

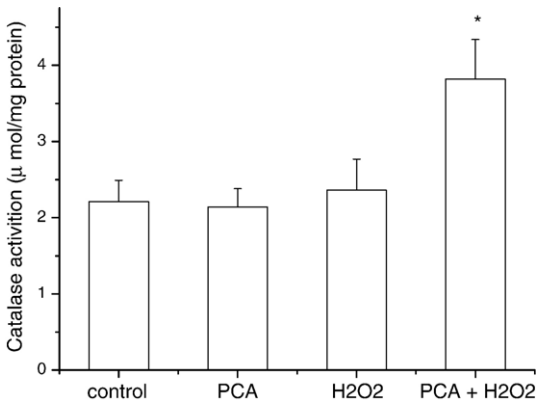

Fig. 6. Effects of PCA from *A. oxyphylla* on catalase activity in H_2O_2-induced PC12 cells. Cells (1×10^5 cells/ml) were treated with 0.4 mM H_2O_2 in the absence or presence of 1.2 mM PCA for 24 h at 37 °C. Data are mean ± S.E.M. values obtained from three culture wells per experiment, determined in three independent experiments. (*) $P<0.05$ statistically different from H_2O_2-induced group.

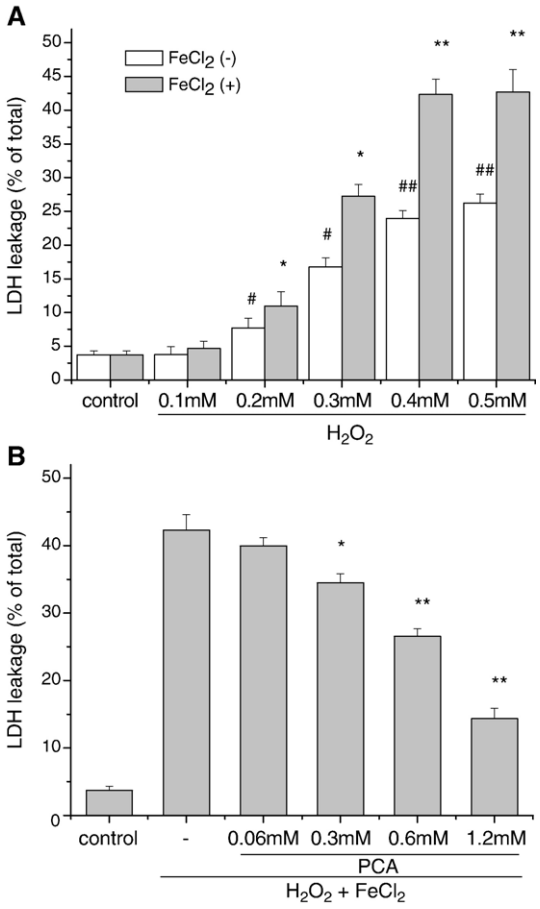

Fig. 7. Effects of PCA from *A. oxyphylla* on H_2O_2/Fe^{2+}-induced PC12 cell damage. (A) Cells (1×10^5 cells/ml) were exposed to various concentration of H_2O_2 in the absence or presence of 60 μM $FeCl_2$ for 12 h. Data are mean±S.E.M. values obtained from three culture wells per experiment, determined in three independent experiments. ($^{\#}$) $P<0.05$ and ($^{\#\#}$) $P<0.01$ in comparison with control; (*) $P<0.05$ and (**) $P<0.01$ statistically different from $FeCl_2$ (−) group. (B) PC12 cells (1×10^5 cells/ml) were treated with 0.4 mM H_2O_2 and 60 μM $FeCl_2$ in the absence or presence of PCA. Data are mean±S.E.M. values obtained from three culture wells per experiment, determined in three independent experiments. (*) $P<0.05$ and (**) $P<0.01$ statistically different from indicated ($H_2O_2+FeCl_2$) group.

Fig. 6, PCA or H_2O_2 alone did not affect enzyme activity, whereas treatment with 1.2 mM PCA significantly augmented the activities of catalase in the cells exposed to H_2O_2 for 24 h. On the other hand, cellular glutathione peroxidase activity was unaffected by H_2O_2 with or without PCA treatment (data not shown).

3.4. Effects of PCA from A. oxyphylla on H_2O_2/Fe^{2+} induced PC12 cell damage

Toxicity of H_2O_2 in biological systems is largely the result of interaction of H_2O_2 with Fe^{2+} to yield OH-like species. The effect of PCA from *A. oxyphylla* on the generation of free radicals during the Fenton reaction ($H_2O_2+Fe^{2+}$) was evaluated. When PC12 cells were exposed to various concentration of H_2O_2 in the presence of Fe^{2+}, leakage of LDH into the culture medium was markedly enhanced (Fig. 7A). As shown in Fig. 7B, PCA similarly suppressed LDH leakage from cells exposed to H_2O_2 and Fe^{2+} in a dose-dependent manner.

4. Discussion

A great number of studies have shown that antioxidants — both endogenous and dietary — can protect nervous tissue from damage by oxidative stress (Contestabile, 2001). Oxidative stress is the harmful condition that occurs when there is an excess of reactive oxygen species and/or a decrease in antioxidant levels. Therefore, removal of excess reactive oxygen species or suppression of their generation by antioxidants may be effective in preventing oxidative cell death. Recently, researchers have made considerable efforts to search for natural substances for the neuroprotective potential, attention has been focused on a wide array of dietary antioxidants that can scavenge free radicals and protect cells from oxidative damage. PCA, a phenolic compound, occurs naturally in many Chinese herbal medicines such as *Salvia miltiorrhiza* (Danshen) (Chan et al., 2004) and *Hibiscus sabdariffa* L. (Liu et al., 2002). In the previous study, PCA was isolated from the kernels of *A. oxyphylla* for the first time and has significant effect on inhibiting the 1-methyl-4-phenylpyridinium ion (MPP^+)-induced neurotoxicity in cultured PC12 cells (An et al., 2006). The present findings demonstrate that H_2O_2-induced apoptotic death via oxidative stress in cultured PC12 cells was also reduced by PCA. PCA from *A. oxyphylla* (over 0.3 mM in concentration) increased PC12 cellular viability and showed significant protective effect against H_2O_2-induced cytotoxicity in PC12 cells. Increased LDH leakage and decreased viability in differentiated PC12 cells exposed to H_2O_2 in the presence or absence of Fe^{2+} was significantly attenuated by treatment with PCA.

Reactive oxygen species readily damage biological molecules, which can ultimately lead to apoptotic or necrotic cell death (Gorman et al., 1996; Gardner et al., 1997; Fiers et al., 1999). For instance, H_2O_2 can induce apoptosis in many different cell types (Whittemore et al., 1994, 1995; Deng et al., 1999), and this effect can be blocked by addition of antioxidants (O'Brien et al., 2000; Behl, 2000; Jang and Surh, 2001). Therefore, the effect of PCA on H_2O_2- induced apoptosis was investigated by flow cytometer analysis. The cells exposed to H_2O_2 exhibited distinct increase in the percentage of cells with a sub-G1 DNA content, which is representative to programmed cell death. In contrast, treatment with PCA significantly reduced the characteristic of apoptotic cells. The result suggested that PCA protected H_2O_2-induced apoptosis.

The protective effect of PCA against H_2O_2-induced apoptotic cell death was associated with the augmented GSH level and activity of catalase. GSH is the most significant component which directly quenches reactive oxygen species and plays major role in xenobiotic metabolism. When an individual is exposed to high levels of xenobiotic, more GSH is utilized for conjugation making it less available to serve as an antioxidant (Meister, 1994; Anderson, 1996). Reduction of the intracellular GSH content enhances oxidative stress and eventually results in cell death (Merad-Boudia et al., 1998; Ibi et al., 1999). In the present work, the levels of GSH reduced in the cells due to H_2O_2 was significantly elevated by PCA treatment. Moreover, PCA

also enhanced the activities of catalase in PC12 cells exposed to H_2O_2, although without any effect was observed on glutathione peroxidase activity. These results suggest that PCA is very effective in preventing oxidative stress triggered by deterioration of cellular functions to reduce the levels of reactive oxygen species. In addition, the cytoprotective role of PCA was also shown in the cells exposed to H_2O_2 and Fe^{2+}. In the presence of Fe^{2+}, OH generated by the Fenton reaction appeared to elicit higher levels of LDH leakage than H_2O_2 itself. In contrast with the role of PCA against H_2O_2-induced cell injury, the effect of PCA against OH generated by the Fenton reaction was not specific. It is well known that there are two types of antioxidants that act against OH. One suppresses their generation, and the other scavenges OH. The mechanism of antioxidant action of plant phenolics (Bahorun et al., 1996; Periera da Silva et al., 2000; Czinner et al., 2001; Lodovici et al., 2001a,b), among them phenolic acids (Croft, 1998; Morton et al., 2000) has usually been attributed to OH scavenging activity. On the other hand, some results indicate that phenolic acids (including PCA) may sequester iron from reacting with H_2O_2 to generate OH, rather than directly scavenging OH. The protective effect of these compounds should be attributed to their capability to bind iron (Ueda et al., 1996; 300; Exner et al., 2000; Lodovici et al., 2001a,b). At present, the cellular and molecular mechanisms that underlie the action of PCA are not fully understood. Our results show that the antioxidant effect may be a major mechanism for PCA-mediated neuroprotection. Further studies of antioxidant and antiapoptotic mechanism of PCA in detail will be required to elucidate before definite conclusions can be drawn.

In conclusion, PCA from *A. oxyphylla* promoted PC12 cell viability, inhibited H_2O_2-induced apoptotic cell death, augmented GSH level and enhanced the activity of catalase. Therefore, PCA may be a candidate chemical for the treatment of oxidative stress-induced neurodegenerative disease.

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