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A water extract of *Curcuma longa* L. (Zingiberaceae) rescues PC12 cell death caused by pyrogallol or hypoxia/reoxygenation and attenuates hydrogen peroxide induced injury in PC12 cells

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Abstract

A number of studies indicate that free radicals are involved in the neurodegeneration in Alzheimer's disease (AD). The role of superoxide anion ($O_2^{\bullet -}$) in neuronal cell injury induced by reactive oxygen species (ROS) was examined in PC12 cells using pyrogallol (1,2,3-benzenetrior), a donor to release $O_2^{\bullet -}$. Pyrogallol induced PC12 cell death at concentrations, which evidently increased intracellular $O_2^{\bullet -}$, as assessed by $O_2^{\bullet -}$sensitive fluorescent precursor hydroethidine (HEt). A water extract of *Curcuma longa* L. (Zingiberaceae) (CLE), having $O_2^{\bullet -}$ scavenging activity rescued PC12 cells from pyrogallol-induced cell death. Hypoxia/reoxygenation injury of PC12 cells was also blocked by CLE. The present study was also conducted to examine the effect of CLE on H_2O_2-induced toxicity in rat pheochromocytoma line PC12 by measuring cell lesion, level of lipid peroxidation and antioxidant enzyme activities. Following a 30 min exposure of the cells to H_2O_2 (150 μM), a marked decrease in cell survival, activities of glutathione peroxidase and catalase as well as increased production of malondialdehyde (MDA) were found. Pretreatment of the cells with CLE (0.5–10 μg/ml) prior to H_2O_2 exposure significantly elevated the cell survival, antioxidant enzyme activities and decreased the level of MDA. The above-mentioned neuroprotective effects are also observed with tacrine (THA, 1 μM), suggesting that the neuroprotective effects of

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cholinesterase inhibitor might partly contribute to the clinical efficacy in AD treatment. Further understanding of the underlying mechanism of the protective effects of these radical scavengers reducing intracellular $O_2^{\bullet -}$ on neuronal cell death may lead to development of new therapeutic treatments for hypoxic/ischemic brain injury.
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Keywords: Cell death; PC12 cells; Superoxides; Free radical scavengers; Pyrogallol; Hypoxia/reoxygenation; Cholinesterase inhibitor; Free radicals; Hydrogen peroxide; Glutathione peroxidase; Catalase; PC12 cells; Malondialdehyde

Introduction

There are many reports on the pharmacological effects of *Curcuma* drugs such as their antitumor (Khar et al., 1999), anti-inflammatory (Ozaki, 1990), and immunological activities (Gonda et al., 1993). Traditionally, *Curcuma* drugs have been used for Oketsu (various syndromes caused by the obstruction of blood circulation such as arthralgia, psy-chataxia and dysmenorrhea) in the system of Chinese medicine (Li, 1977). Although these drugs used to be thought to have different effects, it is doubtful whether the botanical origins of drugs with these names correspond to those of present day drugs with the same names (Sasaki et al., 2003). Since ancient times, especially for *Curcuma* drugs, it has been difficult to identify the botanical origins, because *Curcuma* plants and drugs are similar in morphology and the naming of drugs has varied, depending on the portion used or the producing area in addition to the botanical origin. Nowa-days, four *Curcuma* drugs are prescribed in Chinese Pharma-copoeia; Yujin (the tubers of *Curcuma wenyujin*, *C. longa*, *C. kwangsiensis* or *C. phaeocaulis*) is said to promote circulation of Qi and eliminate stagnant blood; Jianghuang (the rhizome of *C. longa*) and Pian-Jianghuang (the rhizome of *C. wenyujin*) are said to improve blood stasis and promote the circulation of Qi; and Ezhu (the rhizomes of *C. phaeocaulis*, *C. kwangsiensis* or *C. wenyujin*) is said to promote circulation of Qi and improve blood stasis (Li, 1977). *Curcuma* drugs cannot be simply classified by botanical origin, and differences in the effects among different *Curcuma* drugs are not obvious.

Of them, turmeric *Curcuma longa* L. (Zingiberaceae) is a well-known indigenous herbal medicine. It has long been used as a naturally occurring medicine for the treatment of inflammatory diseases. The in vivo activity of *C. longa* in antidepression may mediated in part through MAO A inhibition in mouse brain (Yu et al., 2002). It was also reported that *C. longa* L. (Zingiberaceae) protected PC12 rat pheochromocytoma and normal human umbilical vein endothelial cells from amyloid β-peptide (amino acid sequence of 1–42) (Aβ) insult. *C. longa* may be protecting the cells from Aβ insult through antioxidant pathway (Kim et al., 2001). As a compound of *C. longa*, curcumin is a natural phenolic compound and is a well-known component of yellow-orange dye derived from the rhizome of the plant *Curcuma longa.* Curcumin (difleruloyl methane). It is a potent anti-tumor agent having anti-inflammatory and anti-oxidant properties. It induces apoptosis in cancer cells (Jaruga et al., 1998; Khar et al., 1999) and inhibits TPA-induced protein kinase C (PKC) activity (Lin et al., 1997). It has also shown anti-bacterial, anti-fungal and anti-trypnosomal activity (Apisariyakul et al., 1995; Dahl et al., 1994; Nose et al., 1998).

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by a progressive loss of cognitive function. Until now, there has been no conclusive hypothesis to unify the enormous number of neuropathological and neurochemical findings. One of the major neuropathological findings in AD is an abnormal extracellular accumulation of Aβ, the major component of senile

plaques (Selkoe, 1994). In recent years, multiple lines of evidences have suggested that free radicals were involved in Aβ induced cytotoxity. A was demonstrated to generate free radicals, induce intracellular reactive oxygen species (ROS) production, cause protein oxidation and lipid peroxidation, these findings implicated that the brain of AD patients are under increased oxidative stress (Behl, 1999). Therapeutic efforts aimed at removal of free radicals or prevention of their formation may be beneficial in AD.

In addition to the accumulation of A in AD brains, deficits in cholinergic neurotransmitter system have been observed (Nordberg, 1992). Current efforts to develop an effective drug treatment for AD are based in large part upon the consistent finding that patients with this disorder suffer from marked reduction of cholinergic neuronal function, resulting in a deficiency in acetylcholine concentration in the central nervous system (Coyle et al., 1983; Krall et al., 1999; Tang and Han, 1999). Cholinergic enhancement strategies have been at the forefront of efforts to pharmacologically palliate the cognitive impairments. So far, cholinesterase inhibitors (ChEIs) are the most popular strategies for increasing cholinergic activity in the brain, and show the most encouraging results as palliative therapy for AD (Tang and Han, 1999; Pomponi et al., 1990). CLE and tacrine (THA) as a well-known inhibitor.

Reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), nitric oxide (NO), superoxide anion ($O_2^{\cdot -}$) and hydroxyl radical ($^{\bullet}OH$) have recently been implicated in the regulation of many important cellular events including transcription factor activation (Schreck et al., 1991), gene expression (Lo and Cruz, 1995), and cellular proliferation (Murrell et al., 1990). In contrast, excessive production of ROS gives rise to activation of events, which lead to death in several types of cells (Wolfe et al., 1994). In fact, ROS are reported to induce death of culture cells in vitro such as cultured neurons (Ratan et al., 1994) and pheochromocytoma PC12 cells (Vimard et al., 1996). On the other hand, cells also possess antioxidant systems to control the redox state, which is important for their survival. In this context, several studies indicate that the levels of intracellular ROS may play an essential role as signaling molecules regulating cell death and survival (Li et al., 1997). However, the precise mechanisms involved in cell death induced by ROS remain an open question and the protective effect mediated by some antioxidants has been still controversial. H_2O_2 is often used to investigate the mechanism of ROS-induced cell death (Kitamura et al., 1999; Goldshmit et al., 2001). The mechanisms of hypoxia- and H_2O_2- induced PC12 cell death has been examine (Yoshimura et al., 1998; Yamakawa et al., 2000). In the biological systems, partial reduction of oxygen occurs, resulting in the generation of ROS. The sequential reduction of oxygen leads to the generation of $O_2^{\cdot -}$, H_2O_2 and $\cdot OH$. However, the role of $O_2^{\cdot -}$ in neuronal cell death has not been fully evaluated.

Pyrogallol has been reported as an $O_2^{\cdot -}$ generator (Yamada et al., 2003). This catechin compound is often used to investigate the role of $O_2^{\cdot -}$ in the biological system (Saeki et al., 1999). Pyrogallol induces $O_2^{\cdot -}$ mediated death of several types of cells such as mesangial cells, human lymphoma cells, and human glioma cells (Yamada et al., 2003). The treatment of PC12 cells with pyrogallol gave rise to increase in intracellular levels of $O_2^{\cdot -}$ which was followed by LDH release into the culture medium.

In the present study, the protective effects of broad-spectrum caspase inhibitors and ROS scavengers were evaluated. Our results indicate that pyrogallol produce $O_2^{\cdot -}$ in the cells, which leads to cell death. In this $O_2^{\cdot -}$ induced PC12 cell death, caspase inhibitors were ineffective to rescue cells. In contrast, ROS scavengers, CLE have a certain protective effect against $O_2^{\cdot -}$ stress. Similarly, in our hypoxic/reoxygenation PC12 cell system, CLE as a $O_2^{\cdot -}$ scavenger rescued cells from death. CLE protected H_2O_2-induced injury when the effects of CLE were examined on H_2O_2-induced injury in PC12 cells. The possible protective mechanisms of these agents will be discussed.

Materials and methods

Materials

Dulbecco's modified Eagle's medium (DMEM), penicillin, horse serum and streptomycin were obtained from Life Technologies (Grand Island, NY, USA). Fetal bovine serum (FBS) was purchased from Irvine Scientific (Santa Ana, CA, USA). The superoxide-releasing agent, pyrogallol (1,2,3-benzenetriol) was purchased from Aldrich (Milwaukee, WI, USA). CLE as the cell permeable $O_2^{\bullet -}$ scavenger was drawing out. Hydroethidine (HEt) was from Molecular Probes (Eugene, OR, USA). Hanks' Balanced salt solution (HBSS) was purchased from Bio Whittaker (Walkersville, MD, USA). The lactate dehydrogenase (LDH) assay kit was from Kyokuto (Tokyo, Japan). Other chemicals were of the highest quality available.

CL extract preparation

The water extracts were prepared as follows. One hundred grams of powdered each drug was refluxed in 1 l of H_2O for 2 h. After cooling down to room temperature, the solution was centrifuged at 1200 g for 15 min, filtered and freeze-dried into a resultant powder. Only the extract of *C. longa* (CLE) was purified two times, because it contained many lipophilic compounds. The precipitate was dissolved in water and freeze-dried into whitish powder. The yield of extracts was 13 g (13.0%). For the present experiments, water extracts was dissolved in distilled water.

Cell culture

A PC12 cell line was pure stock (KCTC, Daejon, Korea). PC12 cells were also high passages from ATCC (American Type Culture Collection) and maintained at 37°C in a humidified atmosphere containing 5% CO_2. The cells were grown in 100-mm-diameter tissue culture dishes with culture medium composed of DMEM supplemented with 10% (v/v) FBS, 5% (v/v) horse serum, 100 U/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere containing 5% CO_2 at 37°C. For the pyrogallol treatment, the cells (2.5×10^5 cells per ml) were incubated in 60-mm-diameter dishes for 48 h and the treatment was initiated by replacing the medium with fresh medium containing various concentrations of pyrogallol. For the hypoxia/reoxygenation study, hypoxia was induced in a multigas incubator (MCO-175M; SANYO, Osaka, Japan) (Saeki et al., 1999). In brief, the cells (2×10^5 cells per ml) were first incubated in medium for 48 h and the medium was then exchanged into DMEM containing 2% (v/v) FBS for 24 h. The culture dishes were then placed into the multigas incubator (set to 1% O_2, with 5% CO_2 and 94% N_2). After hypoxic incubation for 12 h, the medium was replaced with the growth medium and the dishes were returned to a standard normoxic atmosphere (21% O_2, 5% CO_2). When the effects of CLE, the cells were preincubated for 1 h with one of the agents prior to pyrogallol treatment. For the reoxygenation experiments, one of the agents added for the last 1 h during hypoxic incubation for 12 h.

For protection of CLE on hydrogen peroxide (H_2O_2)-induced cell death, cells were seeded into multiwell plates (Greiner) at a density of 2×10^4 cells per ml in phenolsulfonphthalein (phenol red) free RPMI1640 medium (Gibco), supplemented with 10% heat-inactivated bovine calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine. All experiments were carried out 24–48 h

after cells were seeded. Hydrogen peroxide (H_2O_2, 8.8 μM solution) was stored at 4°C until 100 mM stock solutions were prepared in phosphate-buffered saline (PBS) on the day of application to the cultures. The 100 mM H_2O_2 was further diluted for addition to the cultures. CLE (provided by Department of Phytochemistry, this Institute) and THA (Sigma) were all dissolved and diluted with PBS. After pretreatment with testing ChEI for 2 h, hydrogen peroxide was added to PC12 cell cultures for 30 min, then replaced with fresh medium. Assays for cell viability, lipid peroxidation and antioxidant enzyme activities were performed 24 h after cultured in fresh medium (Huperzine, 1996).

Cell viability

Cell survival was evaluated by two different methods: morphological observation with phase-contrast microscope (Nikon) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) reduction. For assay of antioxidant enzymes and lipid peroxide, the cultures were washed with ice cold PBS and then pooled in 0.5% Triton X-100 and 0.1 M PBS-0.05 mM ethylene diamine tetra-acetic acid (EDTA) buffered solution and homogenized. The homogenate was centrifuged for 20 min at 3000 × g at 4°C. The supernatants were used in the assay.

Detection of intracellular $O_2^{\cdot -}$

Intracellular production of $O_2^{\cdot -}$ was measured by using HEt. HEt has been shown to be relatively specific for $O_2^{\cdot -}$. $O_2^{\cdot -}$ is able to oxidize HEt to yield ethidium. The harvested cells were washed with HBSS and then were incubated with 10 μM HEt for 15 min at 37°C. Ethidium fluorescence was measured using a spectrofluorometer (Hitachi F-2000, Japan) with excitation at 473 nm and emission at 593 nm. The results were expressed as a percentage of the fluorescence intensity compared with the non-treated samples.

Analysis of cellular damage

Cell injury was monitored by measuring the leakage of LDH into the culture medium, because loss of cell membrane integrity was observed in both necrotic and terminal apoptotic PC12 cells after exposure to hypoxia (Yamada et al., 2003). Culture medium and cells were harvested into micro tube and were centrifuged at 900 × g for 5 min. Eighty microliter of the resulting supernatant was collected. The activity of LDH was determined spectrophotometrically from the changes in absorbance at 560 nm, using 0.18 mM NADH and 0.72 mM pyruvate as substrates in 50 mM phosphate buffer. As a positive control, the whole cell lysate suspended in phosphate-buffered saline (PBS) containing 1% Triton X-100 solution was used. The amount of released LDH was expressed as percent of total activity in each sample. The second method for evaluating damaged cells, Trypan blue exclusion test was performed. The cells were collected by centrifugation, and stained with Trypan blue (0.4% in PBS). The cells were observed under phase contrast microscopy and the bluely-stained cells were counted among a total of 1000 cells.

Electron microscopy

Cells were immediately fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer. After harvest, cells were further fixed with 1% OsO_4 in cacodylate buffer. The fixed cells were dyhydrated

through graded concentration of ethanol and finally embedded in Epon. Thin sections were cut on Porter-Blum Mt II ultramicrotome and then stained with uranyl acetate and lead citrate. Samples were examined with JEM-1010 electron microscope (JEOL, Tokyo, Japan).

Glutathione peroxidase (GSH-Px) activity

Glutathione peroxidase (GSH-Px) activity was analyzed by Mills's procedure (Mills, 1959). An enzyme unit of activity was defined as a decrease in 1 μM GSH per min after the decrease in 1 μM/min GSH of non-enzymatic reaction was subtracted. The assay of catalase was based on the consumable rate of H_2O_2 measured at =240 nm by spectrophotometer (Beers and Sizer, 1952). The content of malondialdehyde (MDA), a compound that is produced during lipid peroxidation, was determined by using the thiobarbituric acid method (Yagi, 1976). The level of protein in cells was measured by Coomassie blue protein-binding method using bovine serum as standard (Bradford, 1976). All data were expressed as percent of control value ± SD. Statistical comparison was made using Student's t-test.

Statistical analysis

Data was expressed as means ± SD. Statistical significance was assessed by one-way ANOVA, followed by Dunnett's multiple-range test. P values less than 0.01 was considered as significant. Calculations were performed using (Microsoft, Redmond, WA, USA) and the statistical software package (SAS, Cary, NC, USA).

Results

Pyrogallol induced PC12 cell death

Pyrogallol was employed as a generator of $O_2^{\bullet -}$ (Yamada et al., 2003). Pyrogallol-induced PC12 cell death was assessed by the measurement of released LDH into the culture medium (Fig. 1A). Pyrogallol at concentrations more than 0.2 mM caused a time-dependent cell death. After exposure to 0.5 mM pyrogallol, the released LDH rapidly increased up to approximately 72% at 5 h. Mild cell death was observed at 0.2 mM pyrogallol, when approximately 56% of LDH was released at 24 h. However, at 0.1 mM pyrogallol the released LDH level was comparable to that of control cells. Intracellular $O_2^{\bullet -}$ production by pyrogallol was assessed by the $O_2^{\bullet -}$ sensitive fluorescent dye HEt. Since HEt is taken into the cells and is oxidized by $O_2^{\bullet -}$ to fluorescent ethidium (Rothe and Valet, 1990; Narayanan et al., 1997). In PC12 cells, pyrogallol at 0.5 mM gave rise to a sharp and transient increase in intracellular ethidium fluorescence peaking at 4 h (more than 2-fold increase over the control level), which was followed by a rapid decrease (Fig. 1B).

Suppressive effects of CLE on pyrogallol-induced PC12 cell death

To elucidate the involvement of $O_2^{\bullet -}$ in the pyrogallol-induced PC12 cell death, the cells were pretreated for 1 h with a cell-permeable $O_2^{\bullet -}$ scavenger, CLE (1–50 μg/ml) prior to 0.5 mM pyrogallol

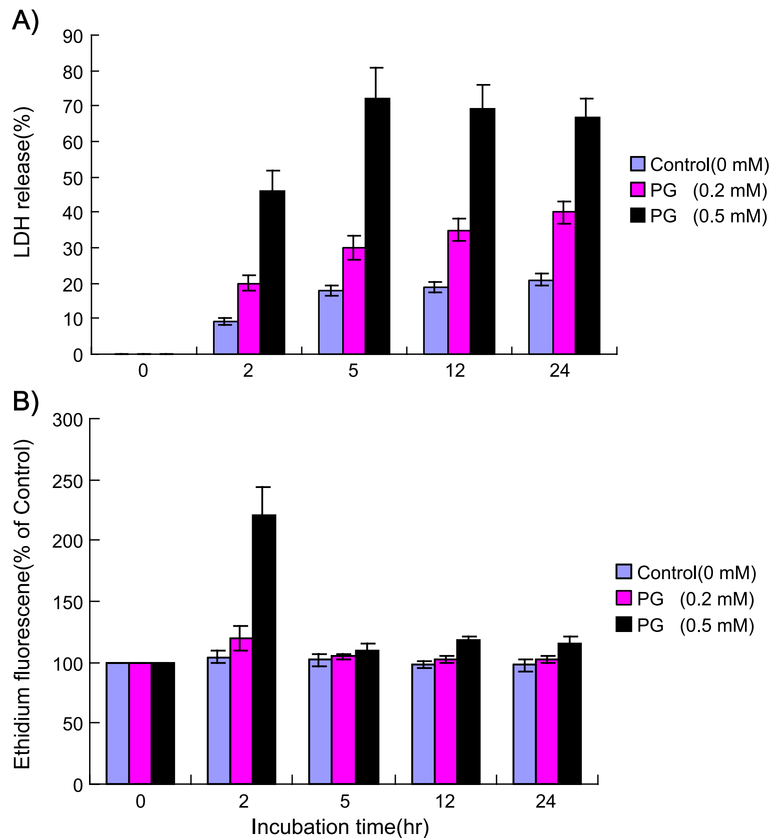

Fig. 1. Time courses of cell death and superoxide production induced by pyrogallol. PC12 cells were exposed to 0.2 mM or 0.5 mM pyrogallol (PG) for the indicated periods of time. (A) Cell death was assessed by LDH release. (B) Superoxide production was assessed in cells loaded with HEt. The data shown are mean ± S.D. from at least three independent experiments, each performed in duplicate or triplicate.

treatment. CLE attenuated concentration-dependent elevation of $O_2^{\cdot -}$ in response to pyrogallol (Table 1A). The LDH assay revealed that CLE significantly decreased pyrogallol-induced PC12 cell death in a concentration-dependent manner (Table 1B). The level of released LDH significantly decreased from 81.7 ± 4.3% (without CLE) to 17.3 ± 2.1% (with 20 μg/ml CLE).

The preventive effect of CLE on cell death were also confirmed by Trypan blue staining. As revealed by LDH assay, the number of Trypan blue-positive cells enormously increased in response to pyrogallol (0.5 mM) (data not shown). CLE drastically decreased the number of Trypan blue-positive cells.

Protective effects of CLE on PC12 cell death by hypoxia/reoxygenation injury

It has been reported that hypoxia/reoxygenation generates superoxide in the neuronal cells (Cazevieille et al., 1993). Scavenging $O_2^{\cdot -}$ by CLE may be effective to protect hypoxia/reoxygenation-induced PC12 cell death. Expectedly, the percentage of LDH release at 5 h after

Table 1
Inhibitory effects of CLE on superoxide production and cell death induced by pyrogallol

A) Superoxide production						
PG	–	+	+	+	+	+
CLE (μg/ml)	–	–	1	5	20	50
Ethidium fluorescence (% of Control)	100 ± 5.4	220 ± 25	211 ± 19	170 ± 21	135 ± 15*	119 ± 17**
B) Cell death						
PG	–	+	+	+	+	+
CLE (μg/ml)	–	–	1	5	20	50
LDH release (%)	24 ± 1.3	68 ± 7.5	54 ± 6.5*	32 ± 2.4**	22 ± 1.5**	20 ± 1.8**

PC12 cells were pretreated with the indicated concentrations of CLE for 1 h, and then exposed to 0.5 mM pyrogallol (PG) for 2 h. (A) Superoxide production was assessed in cells loaded with HEt. (B) Cell death was assessed by LDH release. The data shown are means ± S.D. from two independent experiments, each performed in triplicate. *, P < 0.05; **, P < 0.01 vs. pyrogallol alone.

reoxygenation was decreased by administration of 5–50 μg/ml CLE (Table 2). These results suggest that CLE were effective to reduce cell death by hypoxia/reoxygenation in a concentration-dependent manner.

Effects of CLE and tacrine (THA) on cell viability, MDA level, GSH-Px and catalase activities as cell injury parameter in PC12 cell induced by H_2O_2

As shown in Fig. 2, PC12 cells exhibited a marked decrease in cell number and most cells demonstrated round shape and some of which were lyzed or replaced by debris following 30 min exposure of the cells to 150 μM H_2O_2. Meanwhile, cell viability as determined by MTT reduction was significantly decreased (Fig. 3D), suggesting that PC12 cells were very sensitive to H_2O_2-induced cell injury. However, cultures exposure to the same amount of H_2O_2 in the presence of CLE at concentration of 0.5–10 μg/ml M appeared markedly preserved (Fig. 3D). PC12 cells treated with 150 μM H_2O_2 caused the decrease in the activities of GSH-Px and catalase by 59 and 64%, respectively, ($P < 0.01$), and the level of intracellular MDA, a product of lipid peroxidation, was raised by 51%. In contrast, PC12 cells exposure to the same amount of H_2O_2 in the presence of CLE (0.5–10 μg/ml) markedly attenuated the decrease in GSH-Px and catalase activities (Fig. 3A,C) as well as the increase in MDA level (Fig. 3B). The same protective effects were also found with THA (1 μM).

Table 2
Effects of CLE on cell death induced by hypoxia/reoxygenation

	CLE (μg/ml)				
	0	1	5	20	50
LDH release (%)	17 ± 1.4	12 ± 1.1	8 ± 0.5*	6 ± 0.2**	5 ± 0.2**

PC12 cells were exposed to hypoxia (1% O_2) for 12 h, and treated with the indicated concentrations of CLE for the last 1 h. Then, cells were immediately returned to a normoxic atmosphere. After normoxic incubation for 6 h, cells were harvested and cell death was assessed by LDH release. The data shown are means ± S.D. from two independent experiments, each performed in triplicate. *, P < 0.05 and **, P < 0.01 vs. hypoxia/reoxygenation alone.

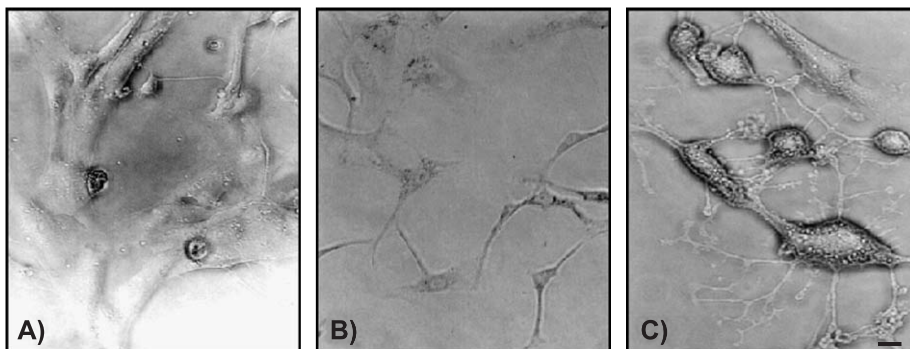

Fig. 2. Effects of CLE on PC12 cell injury induced by H_2O_2. (A) PC12 control cells. (B) PC12 cells exposed to 150 μM H_2O_2 for 30 min. There is a significant decrease in cell number and most of cells demonstrated round shape. (C) PC12 cells were preincubated with 1.0 μg/ml CLE and exposed to 150 μM H_2O_2 for 30 min. All photos were taken 24 h after exposure to H_2O_2. Scale bar, 10 m.

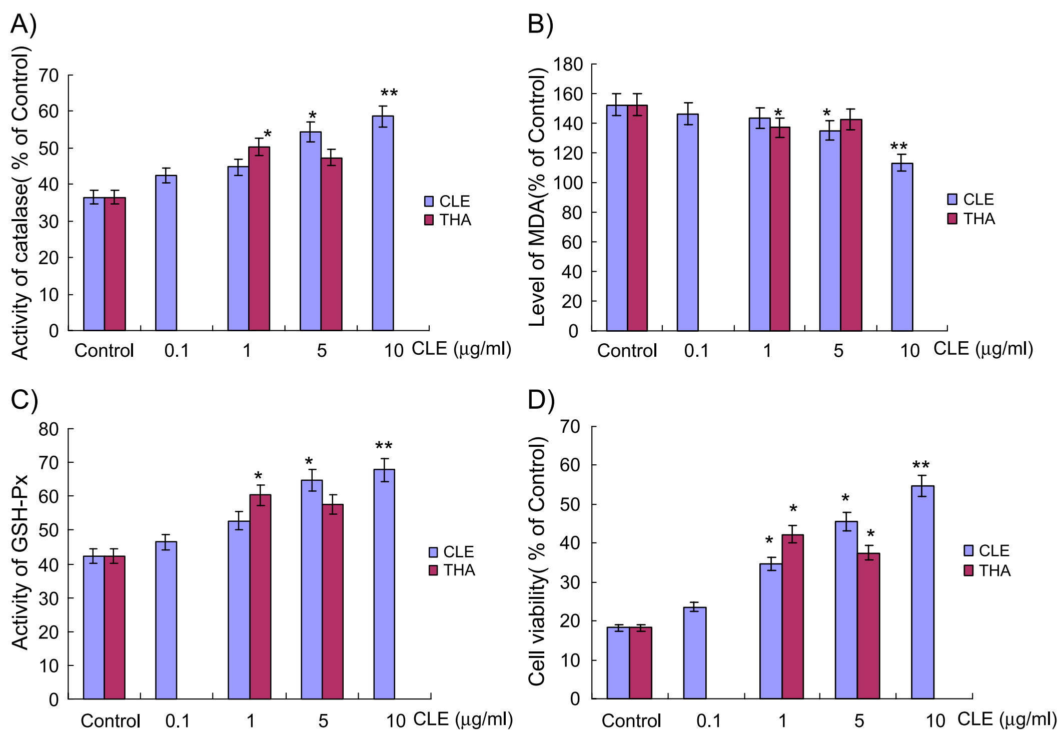

Fig. 3. Effects of CLE and tacrine (THA) on cell viability, MDA level, GSH-Px and catalase activities in PC12 cell. Control activity for GSH-Px and catalase were 11.23±0.3 and 18.7±0.4 (U/mg protein), the concentration of MDA is 1.8 ± 0.5 (nM/mg protein). Cells were incubated with 150 μM H_2O_2 for 30 min. Each acetylcholinesterase inhibitor was added to the culture 2 h prior to H_2O_2 addition. All data were expressed as percent of control value ± s. Statistical comparison was made using Student's t-test. At least two independent experiments were carried out in triplicates. *$P < 0.05$; **$P < 0.01$ vs. H_2O_2 group.

Discussion

Loss of blood flow to the brain gives rise to ischemic injury, which is often associated with the reperfusion of the previously ischemic tissue. The production of ROS is implicated in so-called reperfusion injury. The sequential reduction of oxygen leads to the generation of $O_2^{\cdot-}$ first, followed by H_2O_2 and $^{\cdot}OH$. We focused on cell death induced by pyrogallol to know the physiological and pathological roles of $O_2^{\cdot-}$. Pyrogallol induced production of intracellular $O_2^{\cdot-}$ which was followed by LDH release in PC12 cells. Therefore, it is speculated that PC12 cells stimulated with pyrogallol underwent necrotic cell death. It was shown that pyrogallol induced apoptosis of human glioma cells (Sawada et al., 2001). The modes of cell death may be dependent on types of cells. Recent reports indicate the ROS-dependent necrosis in cortical neurons (Han et al., 2001). Therefore, neuronal cells are readily sensitive to oxidative stresses.

It was shown that PC12 cells stimulated with exogenous H_2O_2 underwent caspase-dependent apoptosis (Yamakawa et al., 2000). H_2O_2, a member of ROS, which locates at downstream of $O_2^{\cdot-}$ apparently induced apoptosis. The observed differences in the types of cell death could be due to the type of ROS employed H_2O_2 and $O_2^{\cdot-}$. However, it is obscure whether exogenously added H_2O_2 directly enters into the cell. H_2O_2 could rather modify the molecules attached and/or integrated in the membrane, which trigger apoptotic signals. In contrast, pyrogallol leads to generation of $O_2^{\cdot-}$ in the cells. This is another reason why we used pyrogallol to investigate the function of ROS. Under hypoxic condition, PC12 cells underwent caspase-dependent apoptotic cell death (Yoshimura et al., 1998).

On the other hand, the most important pathological hallmark of AD is the loss of cholinergic neurons and extracellular deposition of Aβ. The link between cholinergic dysfunction and AD severity provides a firm rationale for the therapeutic usage of cholinesterase inhibitor (ChEI). Indeed, THA has been reported to improve cognitive impairments both in animal models of memory deficiency and AD patients (Pomponi et al., 1990), although the precise mechanisms for these agents remain unclear. The generation of free radical molecules can lead to damage or destructive of a variety of tissue. The major reactive oxidants in cell are $O_2^{\cdot-}$ (superoxide) and the more detrimental hydroxyl radical, the latter being derived from H_2O_2. However, cells are often equipped with several antioxidants. Glutathion peroxidase (GSH-Px), catalase and superoxide dismutase (SOD) mainly serve as detoxifying system to prevent damage caused by reactive oxygen species and play a pivotal role. SOD maintains a very low steady-state intracellular $O_2^{\cdot-}$. The H_2O_2 thus formed is removed by catalase, 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. PC12 cell line, which is employed in our experiment is widely used in vitro model in neuronal injury and oxidative stress (Jackson et al., 1990). The present results showed that H_2O_2-induced decrease in cell survival was correlated with reduction in GSH-Px and catalase activities. When PC12 cells were preincubated with CLE, an elevation in activities of catalase and GSH-Px as well as cell survival were observed, suggesting that cytoprotective effects of the CLE are involved in a stimulation of against intermediate concentrations of H_2O_2-caused PC12 cells injury. It has been reported that Aβ-induced neuron cell death suggested to be direct peroxidation of membrane (Behl, 1999), nicotinic receptor (nAChR) agonist exert neuroprotective properties in cell in Aβ-induced toxicity (Kihara et al., 1998; Svensson and Nordberg, 1999). Recent researches indicate that THA was non-competitive nAChR agonists (Svensson and Nordberg, 1997).

CLE is a less well-known antioxidant, and its effects on neuronal cells have not extensively been studied, although it is known that CLE contains a cell-permeable low-molecular-weight phenolic compound and free radical scavenger of intracellular $O_2^{\cdot-}$ and $^{\cdot}OH$ (Lee, 2003). With regard to

neuroprotective effects of CLE, recently, it has been reported that a methanolic extract of turmeric (*Curcuma longa*) protects PC12 cells from Aβ (Kim et al., 2001) and several anti-oxidative molecules such as calebin-A, curcumin, demethoxycurcumin, bisdemethoxycurcumin have been isolated following a bioassay-guided fractionation utilizing an assay to detect protection of PC12 and HUVEC cells from Aβ insult (Park and Kim, 2002). Oral administration of a phenolic antioxidant curcumin has been shown to be centrally neuroprotective (Ono et al., 2004). Recently, Lim et al. (2001) reported that curcumin reduced oxidative damage and amyloid pathology in an Alzheimer transgenic amyloid precursor protein with Swedish mutant mouse model. Furthermore, curcumin significantly suppressed the inflammatory cytokine interleukin-1β and the astrocytic inflammatory marker proteins, reduced oxidative damage, and decreased overall insoluble amyloid, soluble amyloid, and plaque burden (Lim et al., 2001).

In the present study, we have further demonstrated that CLE reduced PC12 cells from death induced by pyrogallol in a concentration-dependent manner. Further understanding of the underlying mechanism of the protective effects of the CLE reducing intracellular $O_2^{\cdot-}$ on neuronal cell death may lead to development of new therapeutic treatments for hypoxic/ischemic brain injury since CLE and THA have the protection against H_2O_2 insult. Their neuroprotective effects against H_2O_2 toxicity might be of important and contribute in part to their clinical efficacy for the treatment of AD. Our results suggest that turmeric may be a potentially valuable source of natural therapeutic agents for the treatment AD patients. We are in progress of developing the antioxidant medicinal plant, *C. longa* (turmeric) into therapeutic agents.

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