Curcumin protects mitochondria from oxidative damage and attenuates apoptosis in cortical neurons[1]

Yuan-gui ZHU[2], Xiao-chun CHEN[3], Zhi-zhe CHEN[4], Yu-qi ZENG, Guang-bin SHI, Yan-hua SU, Xu PENG

Fujian Institute of Geriatrics, [3]Fujian Institute of Hematology, Union Hospital, Fujian Medical University, Fuzhou 350001, China

KEY WORDS Curcumin; *tert*-butyl hydroperoxide; apoptosis; oxidative stress; Bcl-2 family proteins; mitochondria; Alzheimer disease

ABSTRACT

AIM: To investigate the effect of curcumin on *tert*-butyl hydroperoxide (t-BHP)-induced oxidative damage in rat cortical neurons and to explore the possible mechanism. **METHODS:** Primary cultured rat cortical neurons were performed *in vitro* and cell viability was measured by MTT assay. DNA fragmentation was used to evaluate cell apoptosis. Intracellular reactive oxygen species (ROS) and mitochondrial membrane potential ($\Delta\Psi m$) was determined by flow cytometric assay. Cellular glutathione (GSH) content was measured by spectrophotometer. Bcl-2 family proteins, cytochrome c, cleaved caspase-3, and poly (ADP-ribose) polymerase (PARP) were detected by Western blot. **RESULTS:** Exposure of tBHP 100 μmol/L to neurons for 60 min resulted in $\Delta\Psi m$ loss and cytochrome c release from mitochondria and subsequent activation of caspase-3 and PARP cleavation, and cell apoptosis. After removal of tBHP and then further treatment with curcumin (2.5-20 μmol/L) for 18 h, curcumin abrogated $\Delta\Psi m$ loss and cytochrome c release, blocked activation of caspase 3, and altered the expression of Bcl-2 family. Further curcumin treatment also prevented cellular GSH and decreased intracellular ROS generation markedly. Curcumin eventually attenuated tBHP-induced apoptosis in cortical neurons. **CONCLUSION:** Curcumin may attenuate oxidative damages in cortical neurons by reducing intracellular production of ROS and protecting mitochondria from oxidative damage.

INTRODUCTION

Accumulating data indicate that oxidative stress plays a major role in the pathogenesis of Alzheimer's disease (AD). The production of reactive oxygen species (ROS) can occur very early, even before the appearance of symptoms and molecular events (beta-amyloid plaques and neurofibrillary tangles), leading to tissue damage via several different cellular molecular pathways[1]. Antioxidant micronutrients such as vitamin C, β-carotene, and vitamin E may protect neurons from oxidative stress-induced damage. However, natural antioxidants have recently received much attention as promising agents for reducing the risk of oxidative stress-related diseases. Curcumin (Fig 1), a yellow curry spice derived from turmeric, possesses both anti-inflammatory and antioxidant properties[2]. This spice is widely used as a food preservative and herbal medicine in India[3], and the reports showed that the prevalence of

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[2] *Now in* Rush University Medical Center, Chicago, IL 60612, USA. E-mail yuangui_zhu@rush.edu
[3] Correspondence to Prof Xiao-chun CHEN.
Phn 86-591-335-7896, ext 8357. Fax 86-591-337-0393.
E-mail xc-chen329@sohu.com
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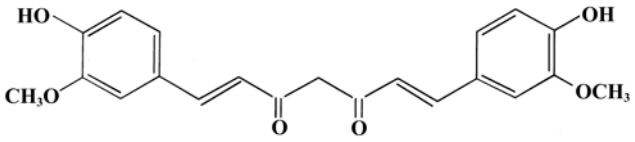

Fig 1. Chemical structure of curcumin [1,7-*bis*-(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione].

AD in patients between 70 and 79 years of age is much less than that of the United States[4]. Recent study also demonstrated that curcumin reduced oxidative damage in an Alzheimer transgenic mouse and showed promise for the prevention of AD[5]. Furthermore, previous studies showed that curcumin was several times more potent than vitamin E as a free radical scavenger, and could protect the brain from lipid peroxidation and scavenge nitric oxide (NO)-based radicals[6,7]. Therefore, it was thought that curcumin might protect neuronal cells from oxidative stress-induced damage.

Apoptosis is a subtype of cell death that is involved in diverse physiological and pathological processes. ROS or other stimuli lead to the release of cytochrome c from mitochondria, which plays a key role in a common pathway of activation of caspases[8]. Activated caspases cleave a variety of target proteins, thereby disabling important cellular processes and breaking down structural components of the cell, and eventually causing cell death. Bcl-2 family of proteins, including pro-apoptotic (Bax, Bad) and anti-apoptotic (Bcl-2, Bcl-xL) proteins, play critical role in the process of apoptosis by controlling mitochondrial permeability and the release of cytochrome c.

The present study was aimed to induce cultured cortical neurons oxidative damage by *tert*-butyl-hydroperoxide (tBHP) and evaluate the protective effect of curcumin. The results showed that curcumin attenuated tBHP-induced apoptosis in cortical neurons by reducing intracellular production of ROS and protecting mitochondria from oxidative damage.

MATERIALS AND METHODS

Materials Curcumin, tBHP, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), 2, 7-dichlorofluorescin diacetate (DCFA), and rhodamine 123 (R123) were obtained from Sigma Chemical Co (St Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, and N2 supplements were from Gibco-BRL (New York, NY, USA). Primary antibodies included cytochrome c from BD PharMingen (San Diego, CA, USA), cleaved caspase-3 and poly (ADP-ribose) polymerase (PARP), and Bad from Cell Signaling (Beverly, MA, USA), Bcl-2, Bax, Bcl-xL and β-actin from Neomarkers (Fremont, CA, USA), phosphorylated neurofilament from Chemicon (Temecula, CA, USA), respectively. Western Blot Chemiluminescent Detection System (LumiGLO system) was from KPL (Gaithersburg, MD, USA). All other regents are analytical or cultured grade purity.

Primary cortical neurons culture Primary culture of cortical neurons was performed as described[9]. In briefly, cortical neurons were from embryonic rats (16-18 d) and then were plated onto poly-*L*-lysine coated dishes and maintained in N_2/ DMEM (1:1) medium with 10 % fetal bovine serum. After 72 h, the culture medium was exchanged for serum-free N_2/DMEM (1:1) for 4 d. Under these conditions, cultures typically contain more than 94 % neurons as assessed by staining with antibody directed against phosphorylated neurofilament.

Cell treatments Seven days after initial planting, the cells were treated with 100 μmol/L tBHP for 60 min to induced oxidative damage. After removal of tBHP-treated medium, the cells were rinsed once with serum free medium and then incubated in culture medium containing with various concentrations of curcumin (2.5, 5.0, 10, or 20 μmol/L) for indicated times. Curcumin was first dissolved in dimethyl sulfoxide (Me_2SO) and later mixed with culture medium to give an appropriate concentration of curcumin with a maximum of 0.5 % Me_2SO. And the controls were designed as followed: 0.5 % (v/v) of Me_2SO was alone added to tBHP-treated cells for 18 h, and 20 μmol/L curcumin was alone added to normal cells for 18 h, and normal cells not treated with any test compounds.

MTT assay After treatment of cultured cortical neurons as described, the culture medium was changed to the medium containing 0.5 g/L MTT, and the cells were incubated further for 4 h. Then cells were mixed thoroughly with an equal volume of alcohol. The absorbance of the supernatant was measured at 570 nm.

ROS assay DCFA is a nonfluorescent compound that is oxidized to the fluorescent 2, 7- dichlorofluorescin (DCF) in the presence of oxidants, which could be quantified using flow cytometry. The cultures were incubated with 10 mmol/L DCF-DA for 15 min at 37 ºC, then were rinsed three times with PBS, and the fluorescence of cell was measured with FACScan flow

cytometer. The data were analyzed using CellQuest™ software (Becton Dickinson, FACScan).

ΔΨm assay ΔΨm was assessed by flow cytometer to analyze cells stained with R123. Cells were incubated with 5 μmol/L R123 for 30 min at 25 ºC and harvested by trypsinization, then analyzed by FACScan flow cytometer. At least 10 000 events were evaluated with excitation set at 485 nm and emission monitored at 530 nm. Histograms were analyzed by the CellQuest™ software and the mean fluorescence intensities were obtained. Relative changes in ΔΨm were expressed as percentage compared to normal control group.

Apoptosis assay For quantitative analysis cell apoptosis, the treated cells were digested with 0.25 % trypsin, then were incubated with mixture of acridine orange (AO) and ethidium bromide (EB) for 15 min at room temperature. Ratio of apoptotic cell was evaluated by Olympus fluorescence microscope. For assay of DNA fragmentation, approximately 5×10^6 cells from each experimental condition, were harvested, and the procedure for extraction of DNA was followed as described [10].

Glutathione (GSH) content measurement The intracellular GSH content was measured by the method described by Ibi[11]. In briefly, the cells were lysed and centrifuged for 20 min at 15 000×*g*, and the supernatant was separated from the acid-precipitated proteins by centrifugation at 15 000×*g* for 2 min after 10 % trichloroacetate was added to. Then the supernatant were mixed with 500 μL of PBS (10 mmol/L, pH 7.5) containing 0.5 mmol/L EDTA, 50 μL of NADPH (4 mmol/L), and 100 μL of GSH reductase (6 U/mL) and then incubated for 15 min at 25 ºC. Then the mixture was mixed with 5 mL of 5,5'-dithiobis-(2-nitrobenzoic acid) (10 mmol/L), and its absorbance was measured at 412 nm. Protein levels were determined by the Bradford methods.

Western blot Proteins were extracted by washing with ice-cold PBS and incubating them for 30 min in lysis buffer containing 50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1 % Nonidet P-40, 0.5 % solution deoxycholate, 1 mmol/L phenylmethysulfonyl fluoride and 100 mg/L leupeptin. Cells lysates were centrifuged at 12 000×*g* for 10 min, and the protein concentrations were determined by the Bradford method. Then 30 μg of total cell protein was separated by 12 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. The membranes were incubated with the appropriate antibody (cleaved caspase-3 and PARP, Bax, Bad, Bcl-2, and Bcl-xL) using Western blot chemiluminescence detection system.

Cytochrome c release The cytochrome c release was detected by the method described by Yang *et al*[12]. Approximately 5×10^6 cells were collected by centrifugation and the pellets were washed with ice-cold PBS and resuspended in 100 μL buffer containing 250 mmol/L sucrose, 20 mmol/L HEPES-KOH, pH 7.5, 10 mmol/L KOH, 1.5 mmol/L $MgCl_2$, 1 mmol/L ethylenediaminetetra-acetic acid (EDTA), 1 mmol/L dithiothreitol and 1 mmol/L phenylmethysulfonyl fluoride. The mitochondria-containing fraction was pelleted by centrifugation at 16 000×*g* for 25 min. The supernatant was used for Western blot analysis with a monoclonal antibody to cytochrome c.

Statistical analysis Statistical analysis of the data for multiple comparisons was performed by analysis of variance (ANOVA) followed by Dunnett's test. For single comparisons, the significance of differences between means was determined by *t*-test.

RESULTS

tBHP-induced apoptosis and effect of curcumin Treatment of cortical neurons with 100 μmol/L tBHP for 60 min resulted in cell viability decrease and cell apoptosis, which can be evaluated by AO-EB staining and DNA fragmentation (Fig 2-4). However, after removal of tBHP and then treatment with curcumin (2.5-20 μmol/L) for 18 h, it was showed that curcumin could prevent tBHP-induced cell viability decrease and attenu-

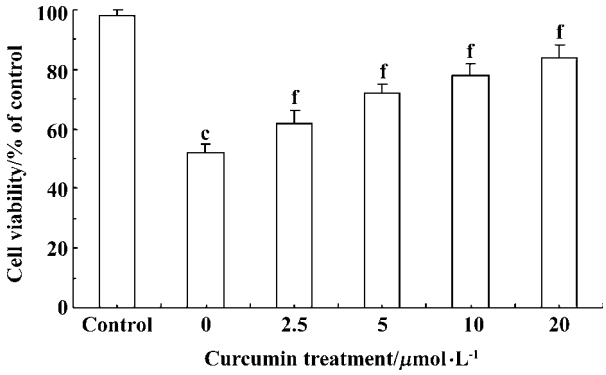

Fig 2. Cell viability of cortical neurons. Cells were exposed to 100 μmol/L tBHP for 60 min, then tBHP was removed and cells were continued to culture with curcumin (2.5-20 μmol/L) for 18 h. $^cP<0.01$ *vs* control. $^fP<0.01$ *vs* without curcumin treatment.

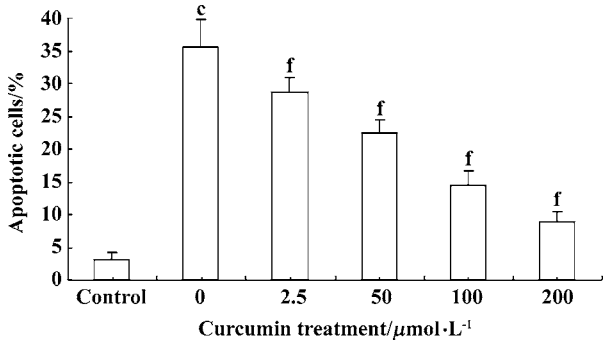

Fig 3. Quantitative apoptotic cortical neurons evaluated by AO/EB staining. Cells were exposed to 100 μmol/L tBHP for 60 min, then tBHP was removed and cells were continued to culture with curcumin (2.5-20 μmol/L) for 18 h. $^{c}P<0.01$ *vs*

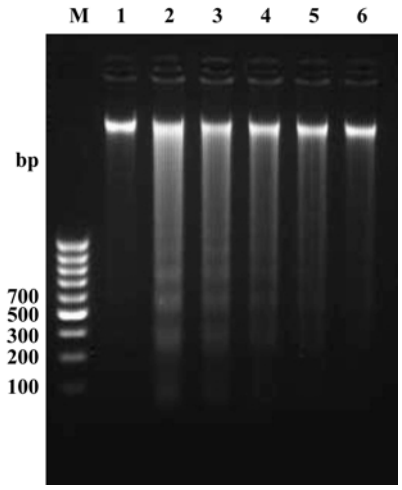

Fig 4. DNA fragmentation of cortical neurons. Cells were exposed to 100 μmol/L tBHP for 60 min, then tBHP was removed and cells were continued to culture with curcumin (2.5-20 μmol/L) for 18 h. Line M:100 bp DNA marker; Line 1 normal, Line 2 to 6 show the concentration of curcumin is 0, 2.5, 5.0, 10.0, 20.0 μmol/L, respectively.

ate cell apoptosis in a concentration-dependent manner (Fig 2, 3). The DNA fragmentation was almost blocked, too (Fig 4). On the other hand, the control results showed only curcumin (20 μmol/L) treatment of cortical neurons for 18 h had no toxic to cell viability (95.6 % ±2.4 %, $P>0.05$) and could not induced cell apoptosis under the evaluation by AO/EB staining (3.6 %±0.8 %, $P>0.05$) or DNA fragmentation (data not shown), and 0.5 % (v/v) Me_2SO had no any protective effect against tBHP-induced oxidative damage on cortical neurons on cell viability (55.6 %± 4.4 %, $P>0.05$) and cell apoptosis (data not shown).

ROS generation and effect of curcumin Treatment of cortical neurons with 100 μmol/L tBHP for 60 min produced a significant increase of ROS (Fig 5). However, after removal of tBHP and then treatment with curcumin (2.5-20 μmol/L) for 18 h, it was showed that curcumin could prevent increase of ROS generation after tBHP insult in a concentration-dependent manner (Fig 5).

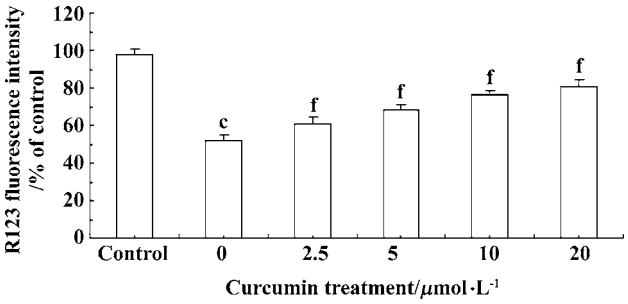

Fig 5. Intracellular reactive oxygen species (ROS) in cortical neurons. Cells were exposed to 100 μmol/L tBHP for 60 min, then tBHP was removed and cells were continued to culture with curcumin (2.5-20 μmol/L) for 18 h. $^{c}P<0.01$ *vs* control. $^{f}P<0.01$ *vs* without curcumin treatment.

ΔΨm loss, cytochrome c release and effect of curcumin After exposure of 100 μmol/L tBHP to cortical neurons for 60 min, then tBHP was removed and cells were continued to be cultured for 1 to 8 h, it was showed that tBHP treatment resulted in ΔΨm loss and cytochrome c release in a time-dependent manner (Fig 6, 7). However, after removal of tBHP and then treatment with curcumin (2.5-20 μmol/L) for 18 h, the curcumin attenuated ΔΨm loss and cytochrome c release (Fig 8, 9). In addition, 20 μmol/L curcumin treatment of cortical neurons alone for 18 h had no obvious changing of ΔΨm (95.6 %± 5.2%, $P>0.05$).

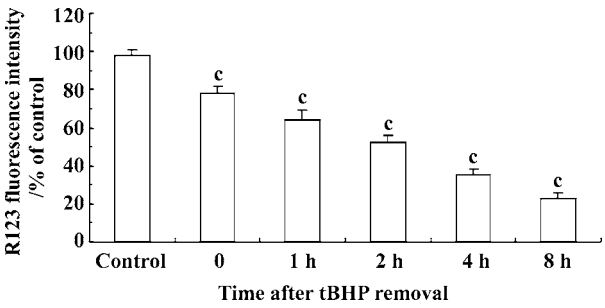

Fig 6. Mitochondrial membrane potential (ΔΨm) in cortical neurons. Cells were exposed to 100 μmol/L tBHP for 60 min, then tBHP was removed and cells were continued to culture with normal culture medium for 1 to 8 h. $^{c}P<0.01$ *vs* control.

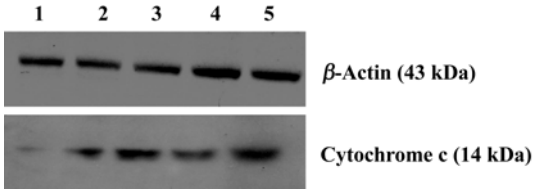

Fig 7. Cytochrome c in cytosol of cortical neurons. Cells were exposed to 100 μmol/L tBHP for 60 min, then tBHP was removed and cells were continued to culture with normal culture medium for 1 to 8 h. Line 1 to 5 is 0, 1, 2, 4, 8 h, respectively.

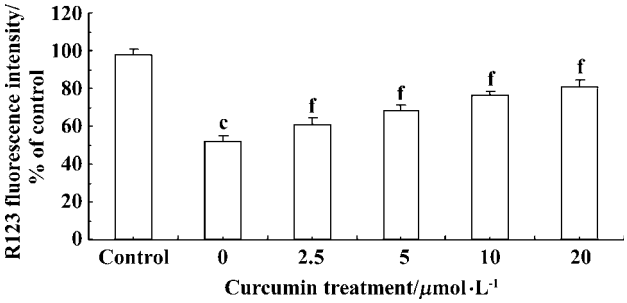

Fig 8. Mitochondrial membrane potential (ΔΨm) in cortical neurons. Cells were exposed to 100 μmol/L tBHP for 60 min, then tBHP was removed and cells were continued to culture with curcumin (2.5-20 μmol/L) for 18 h. $^{c}P<0.01$ *vs* control. $^{f}P<0.01$ *vs* without curcumin treatment.

Effect of curcumin on caspase-3 and PARP activation Treatment of cortical neurons with 100 μmol/L tBHP for 60 min resulted in caspase-3 and PARP activation and cleavation (Fig 9). However, when tBHP was removed and cells were continued to treat with curcumin (2.5-20 μmol/L) for 18 h, the cleavage of caspase-3 and PARP was blocked markedly (Fig 9).

Bcl-2 family proteins and effect of curcumin Treatment of neurons with tBHP decreased the levels of Bcl-2 and Bcl-xL and increased the levels of Bad and Bax obviously (Fig 9), as well as the decrease of ratio of Bcl-2/Bax and Bcl-xL/Bad (data not shown). However, after removal of tBHP and then treatment with curcumin (2.5-20 μmol/L) for 18 h, the levels of Bcl-2 and Bcl-xL was increased and the levels of Bad and Bax was decreased markedly (Fig 9), and the decrease of ratio of Bcl-2/Bax and Bcl-xL/Bad was reversed (data not shown).

Intracellular GSH content and effect of curcumin The basal intracellular GSH content in control primary cultured cortical neurons cultures was 2.89±0.42 μmol/L/100 mg protein. Treatment of

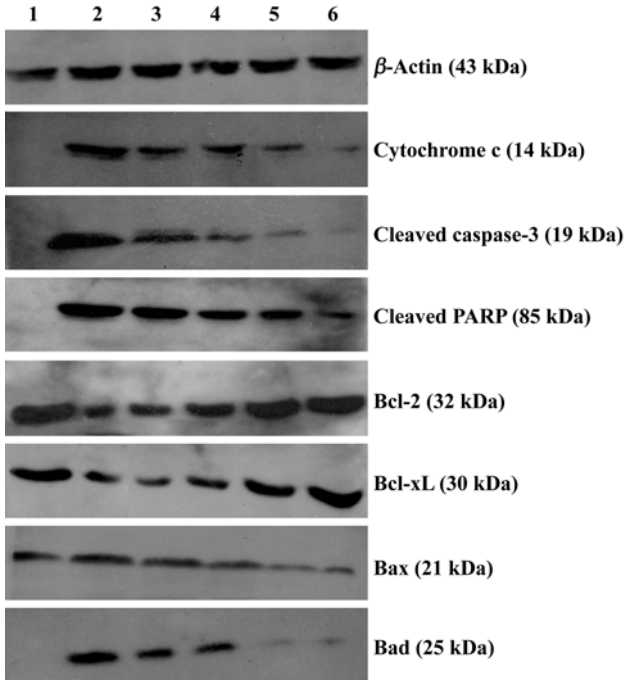

Fig 9. The cytochrome c, cleaved caspase-3 and PARP, Bcl-2 family proteins in cortical neurons. Cells were exposed to 100 μmol/L tBHP for 60 min, then tBHP was removed and cells were continued to culture with curcumin for 18 h. Line 1 normal, Line 2 to 6 show the concentration of curcumin is 0, 2.5, 5.0, 10.0, 20.0 μmol/L, respectively.

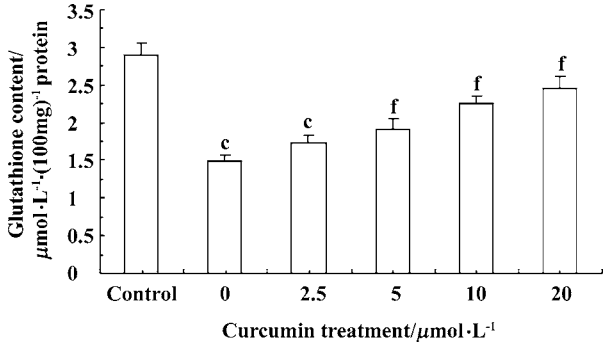

Fig 10. GSH content in cortical neurons. Cells were exposed to 100 μmol/L tBHP for 60 min, then tBHP was removed and cells were continued to culture with curcumin (2.5-20 μmol/L) for 18 h. $^{c}P<0.01$ *vs* control. $^{c}P<0.05$, $^{f}P<0.01$ *vs* without curcumin treatment.

cortical neurons with 100 μmol/L tBHP for 60 min results in intracellular GSH content decrease markedly (Fig 10). However, after removal of tBHP and then treatment with curcumin (2.5-20 μmol/L) for 18 h, it was showed that curcumin significantly increased GSH content (Fig 10).

DISCUSSION

Oxidative stress is critically involved in apoptosis. A growing number of studies have described that ROS, including H_2O_2 and its derived form hydroxyl radical, induced the occurrence of apoptosis[13]. t-BHP is a short chain analog of lipid hydroperoxides which mimics the toxic effect of peroxidized fatty acids. Peroxy radicals can be generated from t-BHP in the cytosol by its interaction with ferrous iron in a reaction similar to the Fenton reaction. t-BHP has been reported to induce apoptosis in the brain *in vivo*[14] and in hepatocytes *in vitro*[15]. The present study also showed t-BHP induced cortical neurons apoptosis. Furthermore, the results also demonstrated that antioxidant curcumin markedly reduced tBHP-induced oxidative damage in cortical neurons.

Curcumin, a major pigment in turmeric obtained from the powdered rhizomes of *Curcuma longa*, possesses both phenolic and β-diketone functional groups, would be expected to have remarkable anti-oxidant and free radical scavenging activities[16]. Curcumin also enhances the activities of anti-oxidant enzymes such as superoxide dismutase, catalase and glutathione peroxidase[17]. Curcumin is also a potent inhibitor of oxygen radical-generating enzymes such as cyclooxygenase-2[18]. Furthermore, GSH plays an important role in protection against oxidative stress-induced injury. The present study also showed that curcumin increased the GSH content of neurons significantly after removal of tBHP. Therefore, it is possible that attenuating the depletion of GSH by tBHP may contribute to the antioxidant activity of curcumin.

Central components of the programmed cell death include caspase cascade, apoptosis proteinase-activating factor-1 (Apaf-1) complex formation, and heterodimerization of Bcl-2 family proteins. Furthermore, cellular organelles, particularly mitochondria, play a crucial role by releasing several apoptotic inducing factors, such as cytochrome c, apoptosis-inducing factor (AIF)[19,20]. During apoptosis, translocation of cytochrome c to the cytosol determines Apaf-1 complex formation. The Apaf-1 complex initially results in activation of caspase-9 followed by the activation of other caspases. Ultimately, caspases chop the cellular proteins resulting in programmed cell death. PARP, a 116 kDa protein, is a substrate for caspases and cleaved during apoptosis to an 85 kDa fragment. Recent studies showed that tBHP leads to cell death by inducing changes in mitochondrial permeability in hepatocytes accompanies by a depolarization of $\Delta\Psi m$[21]. In the present study, the results also showed that tBHP treatment results in $\Delta\Psi m$ loss and cytochrome c release before caspase-3 and PARP activation and DNA fragmentation. Furthermore, antioxidant curcumin can affect the apoptotic process and showed anti-apoptotic actions.

Proteins of the Bcl-2 family play a key role in controlling the activation of caspases[14]. Bcl-2–related proteins fall into two groups that generally either repress apoptosis (Bcl-2 and Bcl-xL) or promote apoptosis (Bax, Bak, and Bad). These proteins influence caspases activation in part by controlling the release of cytochrome c from mitochondria that interacts with the adapter protein Apaf-1, which in turn activates procaspase-9[22]. Pro-apoptotic members like Bax and Bak increase mitochondrial permeability allowing cytochrome c to pass into the cytosol, whereas anti-apoptotic members like Bcl-2 and Bcl-xL prevent cytochrome c release[12,23-25]. In addition, Bcl-2 is also able to regulate activation of membrane-associated procaspase-3 independently of cytochrome *c*[26]. And Bad brings about apoptosis by binding to and inhibiting the antiapoptotic actions of Bcl-xL. In the present study, the results showed when apoptosis occurred, the expression of Bcl-2 and Bcl-xL protein was reduced significantly, while the expression of Bad and Bax was increased. Furthermore, when curcumin attenuated the occurrence of apoptosis, the expression of Bcl-2 family protein was almost reversed to normal condition. These data strongly indicated that Bcl-2 family protein may be involved in the process of curcumin protecting from ROS-induced oxidative damage in cortical neurons.

Taken together, the results presented here provide a mechanism by which oxidative stress provokes death of cortical neurons. In this model, tBHP induced the loss of $\Delta\Psi m$ and the release of cytochrome c from mitochondria and subsequent activation of caspase 3 and PARP cleavation. Curcumin abrogated cytochrome c release or activation of caspase 3 and altered the expression of Bcl-2 family protein and showed protective action on tBHP-induced oxidative damage. Therefore, curcumin can be potentially used as antioxidant for prevention or treatment of Alzheimer's disease and possibly other neurodegenerative diseases with aging.

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