Published in final edited form as:

Toxicol Appl Pharmacol. 2008 April 15; 228(2): 247–255.

Neuronal effects of 4-*t*-Butylcatechol: A model for catechol-containing antioxidants

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Abstract

Many herbal medicines and dietary supplements sold as aids to improve memory or treat neurodegenerative diseases or have other favorable effects on the CNS contain a catechol or similar 1,2-dihydroxy aromatic moiety in their structure. As an approach to isolate and examine the neuroprotective properties of catechols, a simple catechol 4-*t*-butylcatechol (TBC) has been used as a model. In this study, we investigated the effects of TBC on lipopolysaccharide (LPS)-activated microglial-induced neurotoxicity by using the in vitro model of coculture murine microglial-like cell line HAPI with the neuronal-like human neuroblastoma cell line SH-SY5Y. We also examined the effects of TBC on 6-hydroxydopamine (6-OHDA)-induced neurotoxicity in human dopaminergic neuroblastoma SH-SY5Y cells. TBC at concentration from 0.1-10 μ M had no toxic effect on HAPI cells and SH-SY5Y cells, and it inhibited LPS (100 ng/ml)-induced increases of superoxide, intracellular ROS, gp91^{Phox}, iNOS and a decrease of HO-1 in HAPI cells. Under coculture condition, TBC significantly reduced LPS-activated microglia-induced dopaminergic SH-SY5Y cells death. Moreover, TBC (0.1-10 μ M) inhibited 6-OHDA-induced increases of intracellular ROS, iNOS, nNOS, and a decrease of mitochondria membrane potential, and cell death in SH-SY5Y cells. However, the neurotoxic effects of TBC (100 μ M) on SH-SY5Y cells were also observed including the decrease in mitochondria membrane potential and the increase in COX-2 expression and cell death. TBC-induced SH-SY5Y cell death was attenuated by pretreatment with NS-398, a selective COX-2 inhibitor. In conclusion, this study suggests that TBC might possess protective effects on inflammation- and oxidative stress-related neurodegenerative disorders. However, the high concentration of TBC might be toxic, increasing COX-2 expression.

Keywords

4-*t*-Butylcatechol; antioxidant; neurons; microglia; oxidative stress; inflammation

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Introduction

Increasing evidence suggests that reactive oxygen species (ROS) and reactive nitrogen species (RNS) participate in neurodegenerative diseases (Finkel and Holbrook, 2000; Finkel, 2003). Oxidative/nitrosative stress generally describes a condition in which ROS and RNS reach levels where cellular antioxidant defenses are inadequate or overwhelmed (Dalle-Donne *et al.*, 2006). Although evidence from in vitro and in vivo models of neurodegeneration has demonstrated the protective effects of antioxidants, the clinical evidence that antioxidants act as neuroprotective drugs is still relatively controversial (Gilgun-Sherki *et al.*, 2003).

Microglial activation and inflammation-mediated neurotoxicity has been implicated in numerous diseases, including Parkinson's disease (PD), Alzheimer's disease (AD), multiple sclerosis, and AIDS dementia complex (Block *et al.*, 2007). Activated microglia exert cytotoxic effects by oxidative stress (Dickson *et al.*, 1993) releasing inflammatory mediators, such as tumor necrosis factor (TNF)- α , interleukin (IL)-1, nitric oxide (NO), arachidonic acid metabolites, and quinolic acid (Chao *et al.*, 1995). Lipopolysaccharide (LPS) is one of the most common agents used to investigate the impact of inflammation on neuronal death and studies indicate that microglia are necessary for LPS-induced neurotoxicity (An *et al.*, 2002; Qian *et al.*, 2006). LPS is not toxic to neurons, but LPS stimulates glial cells to produce factor(s) that are toxic to neurons (Block and Hong, 2005; Qian *et al.*, 2006; Block *et al.*, 2007). LPS is known to activate protein kinase C, protein-tyrosine kinases, mitogen-activated protein kinase, and NF- κ B, which have been implicated in the release of immune-related cytotoxic factors (Boulet *et al.*, 1992; Bhat *et al.*, 1998; Schumann *et al.*, 1998; Qin *et al.*, 2004).

On the other hand, the progressive deterioration of catecholaminergic cells in Parkinson's patients, at least in part, is due to ongoing selective oxidative stress damage. The auto-oxidation of the neurotransmitter dopamine to 6-hydroxydopamine (6-OHDA) generates ROS and reactive quinones and subsequently induces cell death (Blum *et al.*, 2001; Hanrott *et al.*, 2006). The deleterious consequences of excessive oxidation and the pathophysiological role of ROS have been intensively studied in Alzheimer's disease. Antioxidants are considered a promising approach to slowing the progression and limiting the extent of neuronal cell loss in these disorders (Behl and Moosmann, 2002).

Regardless of the controversy about the efficacy of antioxidant drugs in prevention or treatment of neurodegenerative diseases, antioxidant-containing herbals and dietary supplements are sold as aids to improve memory or treat neurodegenerative diseases or have other favorable effects on the CNS (Chen *et al.*, 2007). Many of the herbal antioxidants contain a catechol or similar 1,2-dihydroxy aromatic moiety in their structure. As an approach to isolate and examine the neuroprotective properties of catechols, a simple catechol 4-*t*-butylcatechol (TBC) has been used as a model catechol-containing antioxidant. It was chosen as a model because its acute and chronic toxicity has been well studied and because of its favorable physical and chemical properties. A 14-week toxicity study in rats and mice exposed to 12500 ppm TBC in the diet resulted in hyperkeratosis of the forestomach probably due to the local irritant effect of the catechol, but little toxicity beyond the stomach (Dunnick, 2002).

In the present study, the effects of TBC on LPS-activated microglia-mediated inflammation and associated secondary neuronal damage were modeled using coculture of the murine microglial-like cell line HAPI with the neuronal-like human neuroblastoma cell line SH-SY5Y. The effects of TBC on 6-OHDA-induced cell toxicity were also examined using SH-SY5Y cells. Our results revealed that concentrations from 0.1 to 10.0 μ M TBC protect against LPS-activated microglia- and 6-OHDA-induced neurotoxicity.

Methods and Materials

Reagents

LPS (L8274) from *Escherichia coli* (O26:B6), 4-*t*-Butylcatechol, dimethyl sulfoxide, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), 2',7'-dichlorodihydrofluorescein diacetate (H₂DCF-DA), NS-398, mouse antibody against iNOS and β -actin were obtained from Sigma Aldrich (U.S.A.). Dulbecco's modified Eagle's medium (DMEM), Ham's F-12 Medium, fetal bovine serum (FBS), penicillin, amphotericin B and streptomycin were obtained from Invitrogen (U.S.A.). Mouse antibody against gp91^{phox} and all materials for SDS-PAGE were obtained from Bio-Rad (U.S.A.). Goat antibody against COX-2, rabbit antibody against nNOS, HO-1, and all horseradish peroxidase-conjugated secondary antibodies were obtained from Santa Cruz Biotechnology (U.S.A.). Enhanced chemiluminescence reagent was purchased from PerkinElmer Life and Analytical Sciences (U.S.A.).

Microglial HAPI Cell Culture and Activated Microglia with LPS

The rat microglia highly aggressively proliferating immortalized HAPI cells, a generous gift from James R. Connor, Ph.D. at Penn State University, M.S. Hershey Medical Center, were cultured in DMEM containing 10% (v/v) heat-inactivated FBS, 4mM glutamine, 100U/ml penicillin, 100 mg/ml streptomycin and 0.25 mg/ml amphotericin B at 37 °C in a humidified incubator under 5% CO₂ and 95% air. For the purpose of experiment, HAPI cells were plated at the density of 1×10^6 cells/ml medium in 24- or 96-well sterile plate. Then, cells were stimulated with LPS alone (100 ng/ml) or LPS with different concentrations of TBC.

Dopaminergic SH-SY5Y Neuronal Cell Culture and Coculture of SH-SY5Y with Microglial HAPI Cells

The effects of TBC on the microglia-mediated, inflammation-associated, secondary neuronal damage, were determined using LPS stimulated HAPI cells as a model of activated microglia (Lin *et al.*, 2007). The human neuroblastoma cell line SH-SY5Y (ATCC CRL-2266) was cultured in a medium consisting of a 1:1 mixture of DMEM and Ham's F-12 medium containing 10% heat-inactivated FBS, 4mM glutamine, 100U/ml penicillin, 100 mg/ml streptomycin and 0.25 mg/ml amphotericin B at 37 °C in a humidified incubator under 5% CO₂ and 95% air. For coculture of the two cell types, SH-SY5Y cells were plated in six-well plates (1×10^6 cells/ml) and HAPI cells were seeded onto cell culture inserts (pore size of 0.2 μ m; NUNCA/S, Roskilde, Denmark), and then placed in the wells where SH-SY5Y cells were growing. The HAPI and SH-SY5Y cocultured cells were separated by filters present in the insert. However, the medium freely passes through the inserts. HAPI cells, then were stimulated with LPS (100 ng/ml) or LPS with TBC.

MTT Cell Viability Assay

Cell viability was measured by a quantitative colorimetric assay with MTT, showing the mitochondrial activity of living cells. In mono-culture, HAPI cells or SH-SY5Y cells in 96-well plates were incubated with 0.01 to 100 μ M TBC for 24 and 48 h, and incubated with LPS (100 ng/ml) for 24 h, respectively. In coculture studies, HAPI cells were treated with or without LPS (100 ng/ml) or LPS + TBC for 24 h. After the incubation, the inserts containing HAPI cells were removed. SH-SY5Y cells were incubated with 50 μ l MTT (final concentration 0.1 mg/ml) for 3 h at 37 °C. The reaction was terminated by addition of 200 μ l DMSO. The amount of MTT product was determined by measuring the absorbance at 560 nm using a microplate reader.

Superoxide Assay

The amount of extracellular superoxide anion ($O_2^{\cdot -}$) produced was determined by measuring the superoxide dismutase-inhibitable reduction of tetrazolium salt, WST-1 (Qin *et al.*, 2004). Cells were plated at 1×10^5 /well in 96-well plates overnight. The cells were washed twice with Hanks' balanced salt solution (HBSS). To each well, 100 μ l of HBSS with or without superoxide dismutase (600 units/ml), 50 μ l of LPS and WST-1 (1 mM) in HBSS were added, respectively. The cultures were incubated for 30 min at 37 °C. The absorbance at 450 nm was read with a Spectra Max Plus microtiter plate spectrophotometer (Molecular Devices, Sunnyvale, CA). The amount of superoxide dismutase-inhibitable superoxide was calculated and expressed as a percentage of untreated control cultures.

Measurement of Intracellular Reactive Oxygen Species

The level of intracellular ROS was quantified by fluorescence with H_2DCF -DA. After incubations with the indicated treatments, microglial HAPI cells or SH-SY5Y cells were loaded with 10 μ M of H_2DCF -DA for 3 h at 37°C. Then, cells were detached from the flask with the addition of 0.25% trypsin–0.02% EDTA, and washed with phosphate-buffered saline, pH 7.4. 10,000 cells were analyzed by a Coulter CyFlow[®] Cytometer (Patrec, Germany). Intracellular ROS-containing cells were identified as those with increased FITC fluorescence of oxidized H_2DCF .

Western Blotting Analysis

After treatment with LPS, or LPS + TBC, HAPI cells were collected and lysed to determine the expression of iNOS, COX-2, gp91^{phox}, and HO-1. SH-SY5Y cells were collected to analyze nNOS, iNOS, and COX-2 expression. The lysates were centrifuged at 15,000g for 30 min at 4°C. The supernatant was collected to use for SDS-polyacrylamide gel electrophoresis. Protein concentration was determined with the Bio-Rad protein assay kit following the manufacturer's guide. Cell membranes were obtained by ultracentrifugation of the supernatant at 26,000g for 1 h at 4°C. Equal amount of protein (20 μ g/lane) were separated by a polyacrylamide gel (10%)-separated protein and transferred to polyvinylidene difluoride membranes from PerkinElmer Life and Analytical Sciences. Nonspecific binding was blocked with 50mM Tris-HCl, pH 7.6, 150mM NaCl, and 0.1% Tween 20 (TBST containing 5% nonfat milk for 1 h at room temperature. The membranes were then incubated overnight at 4°C with one of the following specific primary antibodies: mouse anti-iNOS (1:1000), mouse anti-gp91^{phox} (1:1000), mouse anti- β -actin (1:20,000), rabbit anti-nNOS (1:1000), rabbit anti-HO-1 (1:1000) and goat anti-COX-2 (1:5000). Then membranes were washed six times for 5 min with TBST. The appropriate dilutions of secondary antibodies (diluted 1:1000) were incubated for 1 h. After six washes with TBST, the protein bands were detected with the enhanced chemiluminescence reagent PerkinElmer Life and Analytical Sciences).

Measurement of Mitochondrial Membrane Potential

Mitochondrial membrane potential was measured by the incorporation of a cationic fluorescent dye rhodamine 123. The reduction in fluorescent intensity of rhodamine 123 staining represented a fall in the mitochondrial membrane potential. After 24 h incubation in normal medium with or without treatment, the cells were changed to serum-free medium containing 10 μ M rhodamine 123, and they were incubated for 15 min at 37°C. The cells were then collected, and the fluorescence intensity was analyzed within 15 min by a spectrophotofluorimeter (FLUOstar OPTIMA, BMG LABTECH, Germany, 490-nm excitation and 515-nm emission).

Statistical Analysis

Data were expressed as mean $\pm$ S.E.M. Analysis of variance (ANOVA) was used to assess the statistical significance of the differences followed by Tukey's test for all pair's comparisons. A value of $p < 0.05$ was considered statistically significant. The data were analyzed with the Statistical Package for Social Sciences (SPSS, Chicago, IL).

Results

TBC affected Cells Viability of HAPI cells and SH-SY5Y cells in Concentration- and Time-Dependent Manners

As shown in Fig. 1, TBC at concentrations of 0.1-10 μ M had no effect on the cell viability of HAPI cells (Fig. 1A) and SH-SY5Y cells (Fig. 1B) by MTT assay for 24 and 48h. The extent of MTT reduction in HAPI cells decreased with increasing TBC concentrations. Pairwise comparison of MTT reduction at each concentration by Tukey's test indicated that the increases were significantly different for TBC concentrations of 30 to 50 for 24 h and 30 to 50 for 48 h. Similarly the extent of MTT reduction decreased with increasing TBC concentrations from 50 to 100 for 24 h and 48 h. However, HAPI cells showed no time-dependent changes in MTT reduction, whereas the SH-SY5Y cells showed time-dependent increases in MTT reduction at 50 and 100 μ M of TBC. Therefore, inhibition TBC activated HAPI- or 6-OHDA-induced inflammatory-related responses was not the result of cytotoxicity to HAPI or SH-SY5Y cells. For this reason, the following experimental designs for the protective effects and mechanisms of TBC on LPS-activated microglial HAPI cells- or 6-OHDA-induced neurotoxicity, the concentrations of TBC used were from 0.1-10 μ M.

TBC Inhibited LPS-Activated Microglia HAPI Cells-Induced Loss of SH-SY5Y Cell Viability

In order to examine the effect of TBC (0.1-10 μ M) on LPS-activated microglia-mediated cell viability, coculture of HAPI cells and SH-SY5Y cells was investigated. During coculture, HAPI cells were stimulated with LPS, and viability of SH-SY5Y cells was quantitated by MTT assay. As shown in Fig 2A, LPS (100 ng/ml) caused a decrease in SH-SY5Y cell viability. TBC (1-10 μ M) treatment decreased LPS-activated microglia-induced SH-SY5Y cell death in a concentration dependent manner. In order to clarify that the effect measured occurred in the "SH-SY5Y" side might be due to LPS and TBC traversing the membrane, HAPI cells and SH-SY5Y cells were treated with LPS (100 ng/ml) in monoculture. As shown in Fig. 2B, LPS was not toxic to SH-SY5Y cells at the concentration used.

TBC Reduced LPS-Induced Inflammation and Associated Oxidative Stress-related factors in HAPI Cells

To investigate whether TBC inhibited LPS-activated microglia-induced oxidative stress factors, the effect of TBC on extracellular superoxide, intracellular ROS, and gp91^{phox} on LPS-treated HAPI cells was determined. As shown in Fig. 3, LPS resulted in increases of superoxide (Fig. 3A) and iROS (Fig. 3B) in HAPI cells. The increases of superoxide and ROS release were accompanied by the induction of gp91^{phox} (Fig. 4A). Cotreatment of TBC with LPS reduced the increases of superoxide and intracellular ROS level (Fig. 3) and the overexpression of gp91^{phox} induced by LPS in a concentration dependent manner. (Fig. 4A). As shown in Fig. 4, LPS also increased inflammatory proteins iNOS and COX-2 in HAPI cells. TBC reduced LPS-induced iNOS expression (Fig. 4B). Moreover, HAPI cells incubated with LPS (100 ng/ml) for 15 h significantly increased COX-2 expression (Fig. 4D), and TBC (10 μ M) decreased LPS-induced COX-2 expression. However, LPS was also found to down-regulate HO-1 and down-regulation was prevented by TBC treatment (Fig. 4C).

TBC Decreased Neuronal Death, Intracellular ROS, and Increased Mitochondrial Membrane Potential in 6-OHDA treated SH-SY5Y Cells

As shown in Fig. 5A, exposure of SH-SY5Y cells to 6-OHDA significantly induced cell death. However, treatment with TBC showed **concentration- and time-dependent** effects on cell damage caused by 6-OHDA. SH-SY5Y cells treated with 100 μ M 6-OHDA for 24 h resulted in a significant increase in iROS level (Fig. 5B). TBC inhibited 6-OHDA-induced iROS formation in a concentration-dependent manner. However, when 100 μ M of TBC was used alone, TBC increased iROS (Fig. 5B). Moreover, TBC (0.1 to 10 μ M) also significantly attenuated 6-OHDA-induced decrease in mitochondria membrane potential. However, it was also observed that TBC decreased mitochondrial membrane potential when 100 μ M of TBC was used alone (Fig. 5C).

TBC Attenuated 6-OHDA-Induced Overexpression of nNOS and iNOS in SH-SY5Y Cells

As shown in Fig. 6, 6-OHDA induced up-regulation of nNOS and iNOS in SH-SY5Y cells as revealed by western blot analysis. Results indicated that TBC attenuated 6-OHDA-induced overexpression of nNOS (Fig. 6A) and iNOS (Fig. 6B) in SH-SY5Y cells in a concentration dependent manner.

COX-2 Inhibitor Prevented TBC-Induced SH-SY5Y Cell Death

As shown in Fig. 7A, when SH-SY5Y cells were treated with 100 μ M TBC, a significant increase in COX-2 expression was observed. Therefore, the selective COX-2 inhibitor, NS-398, was used to clarify the relationship of COX-2 and TBC-induced neuronal SH-SY5Y cell death. Results indicated that COX-2 inhibitor pretreatment would attenuate TBC (100 μ M) induced-SH-SY5Y cell death (Fig. 7B). However, TBC also induced SH-SY5Y cell death at concentrations of 30 and 50 μ M (Fig. 1B), but these concentrations of TBC did not increase levels of COX-2 protein (Fig. 7A). NS-398 also did not attenuate TBC (30 and 50 μ M) induced SH-SY5Y cell death (Fig. 7B).

Discussion

The present study demonstrates that TBC protects against LPS-activated microglia- and 6-OHDA-induced neurotoxicity at concentrations of 10 μ M and less. The major findings provided in this study are: (1) TBC (0.1-10 μ M) significantly reduced LPS-activated microglia- and 6-OHDA-induced neuronal cell death. (2) In LPS-activated microglia-mediated toxicity, the protective effect of TBC (0.1-10 μ M) included decreasing the production of superoxide, and iROS, down-regulation of iNOS, COX-2, gp91^{phox}, and up-regulation of HO-1. (3) In 6-OHDA-induced neurotoxicity, the protective effect of TBC (0.1-10 μ M) included down-regulation of nNOS, and iNOS, decreasing accumulation of iROS, and increasing mitochondria membrane potential. (4) Toxicity was observed when 100 μ M TBC was used, which included upregulation of COX-2, iROS, and decrease of mitochondria membrane potential, and increase of neuronal cell death.

The mechanistic basis for the neuroprotective activity of antioxidants does not only rely on the general free radical trapping or antioxidant activity per se in neurons, but also the suppression of genes induced by pro-inflammatory cytokines and other mediators released by glial cells (Wang *et al.*, 2006). The results in this study are consistent with this thesis. TBC is an antioxidant and this study indicated TBC significantly inhibited LPS-induced inflammation and associated neuronal cell damage. Microglial activation and inflammation-mediated neurotoxicity has been implicated in numerous neurodegenerative diseases (Block *et al.*, 2007). Activated microglia exert cytotoxic effects by oxidative stress (Dickson *et al.*, 1993) releasing inflammatory mediators (Chao *et al.*, 1995), including ROS and RNS (Metodiewa and Koska, 2000). The phagocyte NADPH oxidase (PHOX) is a major source of superoxide

and the catalytic center of this oxidase is the membrane-integrated protein gp91^{phox} (Sumimoto *et al.*, 2005; Takeya *et al.*, 2006). Microglia are critical to NADPH oxidase-mediated neurotoxicity in LPS-induced inflammation-mediated neurodegeneration. The mechanism through which NADPH oxidase mediates LPS-induced neurotoxicity was shown to be through ROS-mediated microglial activation and the amplification of microglial proinflammatory gene expression (Qin *et al.*, 2004). As shown in Fig. 8, TBC significantly inhibited LPS-activated microglia-mediated gp91^{phox} activation accompanied with increases of superoxide and iROS. Heme oxygenase (HO) enzymes are important components of cellular antioxidant system. HO-1 has a critical and beneficial role for cells under oxidative stress (Salinas *et al.*, 2003). TBC significantly attenuated LPS-induced down-regulation of HO-1 on microglial HAPI cells. TBC also inhibited LPS-activated microglia-related inflammatory factors, iNOS and COX-2. Therefore, TBC protection against activated microglia-induced neuronal cell death might due to its merits on anti-oxidation and anti-inflammation.

It has been reported that the auto-oxidation of dopamine to 6-OHDA generates ROS and reactive quinones and subsequently induces cell death (Blum *et al.*, 2001; Hanrott *et al.*, 2006). In addition to attenuation of microglia-induced neurotoxicity, TBC also protects against 6-OHDA-induced neuronal cell death. 6-OHDA damages the mitochondrial membrane, resulting eventually in the collapse of mitochondrial membrane potential, thus leading to cell death (Guo *et al.*, 2005). TBC reduced the decrease in mitochondrial membrane potential induced by 6-OHDA. TBC also decreased the overexpression of iNOS and nNOS in 6-OHDA treated neuronal SH-SY5Y cells.

Recent reports have shown that inflammation-mediated COX-2 gene may be an important contributor to neurodegeneration (Rockwell *et al.*, 2000). Brain expresses COX-2 under normal physiology conditions but its levels seem to be dynamically regulated not only by inflammatory signals but also by physiological neuronal plasticity involving, for example, NMDA receptor activation (Yamagata *et al.*, 1993). In neurons, COX-2 has mostly a perinuclear location and is also present in dendritic arborizations and spines of excitatory neurons in the cerebral cortex, hippocampus and amygdala, suggesting that COX-2 products play a role in postsynaptic signaling (Chen *et al.*, 2002). Moreover, neurodegenerative disorders such as Alzheimer's disease and Parkinson's disease were found to be associated with the abnormal accumulation of ubiquitinated proteins (Lowe *et al.*, 2001; Li *et al.*, 2003). Recent reports have shown that proteasome inhibition causes activation of stress-mediating gene and inflammation-mediated COX-2 gene, which may be an important contributor to neurodegeneration (Rockwell *et al.*, 2000). The contribution of COX-2 to mechanisms of neurodegeneration are not well defined. However, epidemiological studies link nonsteroidal anti-inflammatory drugs (NSAID) with delays in the clinical expression of Alzheimer disease (Pasinetti, 1998). The present results indicated that TBC inhibited LPS-induced COX-2 expression. However, our observations also showed that high concentrations of TBC possessed neurotoxicity. High concentrations of TBC induced SH-SY5Y cell death in a concentration- and time-dependent manner. TBC (100 μ M) increased iROS, up-regulated COX-2, and decreased mitochondria membrane potential. Moreover, COX-2 inhibitor NS398 reduced TBC (100 μ M)-induced cell death. Although at 30 and 50 μ M of TBC did not increase the level of COX-2 protein, at least in part, COX-2 up-regulation must play an important role in TBC (100 μ M)-induced toxicity.

In conclusion, this study suggests that the neuroprotective activity of TBC does not only rely on the antioxidant activity per se in neurons, but also on the suppression of inflammatory mediators released by microglia cells (Fig. 8).

Acknowledgements

This research was supported by grant NSC-96-2320-B-039-MY3 from the National Science Council and grant QA096001 from Kaohsiung Medical University, Taiwan; and supported in part by the Intramural Research Program of the National Institutes of Health, National Institute of Environmental Health Sciences, U.S.A..

References

- An H, Xu H, Yu Y, Zhang M, Qi R, Yan X, Liu S, Wang W, Guo Z, Qin Z, Cao X. Up-regulation of TLR9 gene expression by LPS in mouse macrophages via activation of NF-kappaB, ERK and p38 MAPK signal pathways. *Immunol Lett* 2002;81:165–169. [PubMed: 11947920]
- Behl C, Moosmann B. Antioxidant neuroprotection in Alzheimer's disease as preventive and therapeutic approach. *Free Radic Biol Med* 2002;33:182–191. [PubMed: 12106814]
- Bhat NR, Zhang P, Lee JC, Hogan EL. Extracellular signal-regulated kinase and p38 subgroups of mitogen-activated protein kinases regulate inducible nitric oxide synthase and tumor necrosis factor-alpha gene expression in endotoxin-stimulated primary glial cultures. *J Neurosci* 1998;18:1633–1641. [PubMed: 9464988]
- Block ML, Hong JS. Microglia and inflammation-mediated neurodegeneration: multiple triggers with a common mechanism. *Prog Neurobiol* 2005;76:77–98. [PubMed: 16081203]
- Block L, Zecca L, Hong JS. Microglia-mediated neurotoxicity: uncovering the molecular mechanisms. *Nat Rev Neurosci* 2007;8:57–69. [PubMed: 17180163]
- Blum D, Torch S, Lambeng N, Nissou M, Benabid AL, Sadoul R, Verna JM. Molecular pathways involved in the neurotoxicity of 6-OHDA, dopamine and MPTP: contribution to the apoptotic theory in Parkinson's disease. *Prog Neurobiol* 2001;65:135–172. [PubMed: 11403877]
- Boulet I, Ralph S, Stanley E, Lock P, Dunn AR, Green SP, Phillips WA. Lipopolysaccharide- and interferon-gamma-induced expression of hck and lyn tyrosine kinases in murine bone marrow-derived macrophages. *Oncogene* 1992;7:703–710. [PubMed: 1373483]
- Chao CC, Hu S, Peterson PK. Glia, cytokines, and neurotoxicity. *Crit Rev Neurobiol* 1995;9:189–205. [PubMed: 8581983]
- Chen C, Magee JC, Bazan NG. Cyclooxygenase-2 regulates prostaglandin E2 signaling in hippocampal long-term synaptic plasticity. *J Neurophysiol* 2002;87:2851–2857. [PubMed: 12037188]
- Chen LW, Wang YQ, Wei LC, Shi M, Chan YS. Chinese herbs and herbal extracts for neuroprotection of dopaminergic neurons and potential therapeutic treatment of Parkinson's disease. *CNS Neurol Disord Drug Targets* 2007;6:273–281. [PubMed: 17691984]
- Dalle-Donne I, Rossi R, Colombo R, Giustarini D, Milzani A. Biomarkers of oxidative damage in human disease. *Clin Chem* 2006;52:601–623. [PubMed: 16484333]
- Dickson DW, Lee SC, Mattiace LA, Yen SH, Brosnan C. Microglia and cytokines in neurological disease, with special reference to AIDS and Alzheimer's disease. *Glia* 1993;7:75–83. [PubMed: 8423065]
- Dunnick J. NTP technical report on the toxicity studies of p-tert-butylcatechol (CAS No. 98-29-3) administered in feed to F344/N rats and B6C3F1 mice. *Toxic Rep Ser* 2002;5–51. [PubMed: 12592414]
- Finkel T. Oxidant signals and oxidative stress. *Curr Opin Cell Biol* 2003;15:247–254. [PubMed: 12648682]
- Finkel T, Holbrook NJ. Oxidants, oxidative stress and the biology of ageing. *Nature* 2000;408:239–247. [PubMed: 11089981]
- Gilgun-Sherki Y, Melamed E, Offen D. Antioxidant treatment in Alzheimer's disease: current state. *J Mol Neurosci* 2003;21:1–11. [PubMed: 14500988]
- Guo S, Bezard E, Zhao B. Protective effect of green tea polyphenols on the SH-SY5Y cells against 6-OHDA induced apoptosis through ROS-NO pathway. *Free Radic Biol Med* 2005;39:682–695. [PubMed: 16085186]
- Hanrott K, Gudmunsen L, O'Neill MJ, Wonnacott S. 6-hydroxydopamine-induced apoptosis is mediated via extracellular auto-oxidation and caspase 3-dependent activation of protein kinase Cdelta. *J Biol Chem* 2006;281:5373–5382. [PubMed: 16361258]

- Li Z, Jansen M, Pierre SR, Figueiredo-Pereira ME. Neurodegeneration: linking ubiquitin/proteasome pathway impairment with inflammation. *Int J Biochem Cell Biol* 2003;35:547–552. [PubMed: 12672447]
- Lin YC, Uang HW, Lin RJ, Chen IJ, Lo YC. Neuroprotective Effects of GLNVA against Microglia-like Cells and 6-OHDA-Induced Neurotoxicity in SH-SY5Y Human Dopaminergic Neuroblastoma Cells. *J Pharmacol Exp Ther* 2007;323:1–11. [PubMed: 17609422]
- Lowe J, Mayer J, Landon M, Layfield R. Ubiquitin and the molecular pathology of neurodegenerative diseases. *Adv Exp Med Biol* 2001;487:169–186. [PubMed: 11403157]
- Metodiewa D, Koska C. Reactive oxygen species and reactive nitrogen species: relevance to cyto(neuro) toxic events and neurologic disorders. An overview *Neurotox Res* 2000;1:197–233.
- Pasinetti GM. Cyclooxygenase and inflammation in Alzheimer's disease: experimental approaches and clinical interventions. *J Neurosci Res* 1998;54:1–6. [PubMed: 9778144]
- Qian L, Block ML, Wei SJ, Lin CF, Reece J, Pang H, Wilson B, Hong JS, Flood PM. Interleukin-10 protects lipopolysaccharide-induced neurotoxicity in primary midbrain cultures by inhibiting the function of NADPH oxidase. *J Pharmacol Exp Ther* 2006;319:44–52. [PubMed: 16807359]
- Qin L, Liu Y, Wang T, Wei SJ, Block ML, Wilson B, Liu B, Hong JS. NADPH oxidase mediates lipopolysaccharide-induced neurotoxicity and proinflammatory gene expression in activated microglia. *J Biol Chem* 2004;279:1415–1421. [PubMed: 14578353]
- Rockwell P, Yuan H, Magnusson R, Figueiredo-Pereira ME. Proteasome inhibition in neuronal cells induces a proinflammatory response manifested by upregulation of cyclooxygenase-2, its accumulation as ubiquitin conjugates, and production of the prostaglandin PGE(2). *Arch Biochem Biophys* 2000;374:325–333. [PubMed: 10666314]
- Salinas M, Diaz R, Abraham NG, Ruiz de Galarreta CM, Cuadrado A. Nerve growth factor protects against 6-hydroxydopamine-induced oxidative stress by increasing expression of heme oxygenase-1 in a phosphatidylinositol 3-kinase-dependent manner. *J Biol Chem* 2003;278:13898–13904. [PubMed: 12578834]
- Schumann RR, Pfeil D, Freyer D, Buerger W, Lamping N, Kirschning CJ, Goebel UB, Weber JR. Lipopolysaccharide and pneumococcal cell wall components activate the mitogen activated protein kinases (MAPK) erk-1, erk-2, and p38 in astrocytes. *Glia* 1998;22:295–305. [PubMed: 9482215]
- Sumimoto H, Miyano K, Takeya R. Molecular composition and regulation of the Nox family NAD(P)H oxidases. *Biochem Biophys Res Commun* 2005;338:677–686. [PubMed: 16157295]
- Takeya R, Ueno N, Sumimoto H. Regulation of superoxide-producing NADPH oxidases in nonphagocytic cells. *Methods Enzymol* 2006;406:456–468. [PubMed: 16472678]
- Wang JY, Wen LL, Huang YN, Chen YT, Ku MC. Dual effects of antioxidants in neurodegeneration: direct neuroprotection against oxidative stress and indirect protection via suppression of glia-mediated inflammation. *Curr Pharm Des* 2006;12:3521–3533. [PubMed: 17017945]
- Yamagata K, Andreasson KI, Kaufmann WE, Barnes CA, Worley PF. Expression of a mitogen-inducible cyclooxygenase in brain neurons: regulation by synaptic activity and glucocorticoids. *Neuron* 1993;11:371–386. [PubMed: 8352945]

Fig. 1.

Effects of TBC on (A) microglial-like HAPI cells and (B) dopaminergic neuron SH-SY5Y cells. Cells were treated with TBC (0.1-100 μ M) for 24 and 48 h, respectively. Cell viability was determined by MTT assay. Bars represent the mean $\pm$ S.E.M. from six independent experiments. * p <0.05 versus control group (no treatment); #, p <0.05 versus group treated with 24 h; + p <0.05, compared with indicated group; . ANOVA followed by Tukey's test.

Fig. 2.
 (A) Effects of TBC on LPS (100 ng/ml)-activated microglial-like HAPI cells-induced SH-SY5Y cell death. Cells were pretreated with TBC (0.1-10 μM) for 30min, then incubated with LPS for 24 h. (B) Effects of LPS on microglial-like HAPI cells and dopaminergic neuron SH-SY5Y cells. Cell viability was determined by MTT assay. Bars represent the mean ± S.E.M. from six independent experiments. # $p < 0.01$ versus control group (no treatment); * $p < 0.05$ versus LPS only; + $p < 0.05$, compared with indicated group; ANOVA followed by Tukey's test.

Fig. 3. Inhibitory effects of TBC on LPS (100 ng/ml)-induced extracellular superoxide (A) and intracellular ROS formation (B) in HAPI cells. Cultures were pretreated with TBC for 30 min followed by LPS treatment for 30 min. Production of extracellular superoxide was measured as the superoxide dismutase-inhibitable reduction of WST-1. Intracellular ROS were determined by using H₂DCF. Bars represent the mean $\pm$ S.E.M. from six independent experiments. [#] $p < 0.01$ versus control group (no treatment); ^{*} $p < 0.05$ versus LPS only; ⁺ $p < 0.05$, compared with indicated group; ANOVA followed by Tukey's test.

Fig. 4. Inhibitory effects of TBC on LPS (100 ng/ml)-stimulated expressions of gp91^{phox} (A), iNOS (B), HO-1 (C), and COX-2 (D) in HAPI cells. Densitometry analyses are presented as the relative ratio of protein/β-actin protein, and they are represented as percentages of the LPS response in the absence of TBC. Bars represent the mean ± S.E.M. from three independent experiments. #*p*<0.01 versus control group (no treatment); **p*<0.05 versus LPS only; +*p*<0.05, compared with indicated group; ANOVA followed by Tukey's test.

Fig. 5.

(A) Effects of TBC on 6-OHDA (100 μM)-induced SH-SY5Y cell death. Cells were pretreated with TBC (0.1-10 μM) for 30min, then incubated with 6-OHDA for 24 and 48 h, respectively. Cell survival rate was determined by MTT assay. (B) Effects of TBC on 6-OHDA (100 μM)-induced changes of intracellular iROS formation and (C) mitochondria membrane potential in SH-SY5Y cells. The intracellular ROS was measured by using H₂DCF-DA staining and mitochondrial membrane potential was measured by rhodamine 123. Bars represent the mean ± S.E.M. from six independent experiments. #*p*<0.01 versus control group (no treatment); **p*<0.05 versus 6-OHDA only; ϕ*p*<0.05 versus group treated with 24 h; +*p*<0.05, compared with indicated group; ANOVA followed by Tukey's test.

Fig. 6. Inhibitory effects of TBC on 6-OHDA (100 μM)-induced overexpression of nNOS (A) and iNOS (B) in SH-SY5Y cells. Densitometry analyses are presented as the relative ratio of protein/β-actin protein, and they are represented as percentages of the 6-OHDA response in the absence of TBC. Bars represent the mean ± S.E.M. from six independent experiments. ## $p < 0.01$ versus control; # $p < 0.01$ versus control group (no treatment); * $p < 0.05$ versus 6-OHDA only; + $p < 0.05$, compared with indicated group; ANOVA followed by Tukey's test.

Fig. 7.

(A) Effects of TBC on COX-2 expression in SH-SY5Y cells. Cultures were pretreated with TBC for 15 h. Densitometry analyses are presented as the relative ratio of COX-2 protein/β-actin protein, and they are represented as percentages of the control. * $p < 0.01$ versus control group (no treatment). (B) Effects of NS-398, a selective COX-2 inhibitor, on TBC (30-100 μM)-induced cell death. * $p < 0.05$ versus NS-398 untreated group. ANOVA followed by Tukey's test.

Fig. 8.
Hypothetical protective mechanisms of TBC on LPS-activated microglia-like cells- and 6-OHDA-induced neurotoxicity.