Neuroprotective Effect of \textit{trans}-Cinnamaldehyde on the 6-Hydroxydopamine-Induced Dopaminergic Injury

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The anti-inflammatory and neuroprotective effects of \textit{trans}-cinnamaldehyde (TCA) were investigated on the inflammatory cells and the dopaminergic degeneration in mice. TCA inhibited the up-regulation of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) in the lipopolysaccharide (LPS)-induced inflammatory BV2 microglial cells. To investigate the TCA efficacy on the 6-hydroxydopamine (6-OHDA)-induced dopaminergic degeneration in mice, an intracerebroventricular injection of 6-OHDA was given to the mice, and TCA (30 mg/kg) was intraperitoneally administered. At 7 d after the 6-OHDA injection, 6-OHDA led to a severe loss of tyrosine hydroxylase (TH)-positive dopaminergic neurons in the striatum and substantia nigra (SN). On the other hand, TCA dramatically maintained the number of TH-positive dopaminergic neurons in the striatum and SN regions of the 6-OHDA-treated mice, which indicates that TCA is able to inhibit the 6-OHDA-induced reduction of TH expression in the dopaminergic neurons in the striatum and SN regions. TCA also inhibited the induction of iNOS and COX-2 in the 6-OHDA model, similarly as shown in the LPS-induced inflammatory BV2 microglial cells. These results indicate that TCA has a neuroprotective effect on dopaminergic neurons and that this effect may be associated with the inhibition of inflammatory responses. These findings suggest that TCA may be a therapeutic candidate for the prevention of inflammation-mediated neurodegenerative diseases.

Key words: \textit{trans}-cinnamaldehyde; 6-hydroxydopamine; dopaminergic dysfunction; Parkinson’s disease; neurodegeneration

Inflammation, a self-defensive reaction against various pathogenic stimuli, can become a harmful self-damaging process. 1 Accumulating evidence has suggested that inflammatory processes account for the progressive neurodegeneration which is observed in Parkinson’s disease (PD). 2–6 The major pathological changes of PD are the preferential and progressive loss of dopamine (DA)-containing neurons in the substantia nigra pars compacta (SNpc) 7 and the consequent progressive loss of dopamine (DA)-containing neurons in the substantia nigra (SN). On the other hand, TCA dramatically maintained the number of TH-positive dopaminergic neurons in the striatum and SN regions of the 6-OHDA-treated mice, which indicates that TCA is able to inhibit the 6-OHDA-induced reduction of TH expression in the dopaminergic neurons in the striatum and SN regions. TCA also inhibited the induction of iNOS and COX-2 in the 6-OHDA model, similarly as shown in the LPS-induced inflammatory BV2 microglial cells. These results indicate that TCA has a neuroprotective effect on dopaminergic neurons and that this effect may be associated with the inhibition of inflammatory responses. These findings suggest that TCA may be a therapeutic candidate for the prevention of inflammation-mediated neurodegenerative diseases.

\textit{trans}-Cinnamaldehyde (TCA) is one of the main components of \textit{Cinnamomi Ramulus} (CR), young twigs of \textit{Cinnamomum cassia}, which have been widely used for the symptomatic relief of cold pathogenic diseases such as headaches, fever, sweating and pain. 8 Our group recently reported the anti-inflammatory effects of CR 9–13; in addition, some reports have shown that TCA exhibits anti-inflammatory activities.12–16 As cinnamaldehyde showed the anti-inflammatory effect in macrophages, we speculated that TCA may exhibit a similar anti-inflammatory effect in microglia, which act as a sensor for pathological events in the central nervous system (CNS). 17,18 Therefore, we evaluated the effects of TCA on lipopolysaccharide (LPS)-induced nitric oxide (NO) release and the expressions of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) in the BV2 microglial cells. We further investigated the neuroprotective efficacy of TCA on dopaminergic degeneration and its suppressive effects on the increased iNOS and COX-2 expressions, which were induced by a 6-hydroxydopamine (6-OHDA) injection to mice.

\textbf{MATERIALS AND METHODS}

\textbf{Cell Culture and Treatment} BV2 cells (especially derived from the murine brain), an immortalized microglial cell line which is made from microglia which are the resident myeloid-lineage cells in the spinal cord parenchyma and the brain, were grown in Dulbecco’s modified Eagle’s medium (DMEM; Sigma, U.S.A.) containing 5% fetal bovine serum (FBS; Gibco BRL, U.S.A.), 100 U/mL of penicillin, and 100 µg/mL of streptomycin (Gibco BRL, U.S.A.). In all experiments, the cells were treated with the indicated concentrations of TCA (1 or 3 µM, Sigma, U.S.A.) 1 h before the addition of 1 µg/mL of LPS (Sigma, U.S.A.).

\textbf{Cell Viability} Cell viability was measured by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and fluorescein diacetate (FDA)–propidium iodide (PI) staining method. The BV2 microglia cells were seeded at a density of 5000 cells/well in a 96-well plate. The cells were either left behind untreated or stimulated with 1 µg/mL of LPS for 24 h in the absence or presence of TCA (0, 1, or 3 µM). After the LPS treatment, the MTT assay or FDA/PI staining was performed to measure the cell viability. MTT was added to each well and the cells were then incubated for 1 h. After the culture media was discarded, dimethyl sulfoxide (DMSO) was added to dissolve the formazan dye. The optical density was measured at 540 nm. For the FDA/PI staining, cell viability (FDA, green color) and cell death (PI, red color) were

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measured using a fluorescence microscope after the addition of FDA (1 μM) and PI (5 μg/mL).

**Measurement of Nitrite Concentration** NO production was evaluated by measuring the accumulated levels of nitrite in the supernatant 24 h after the addition of LPS using a Griess Reagent (Promega, U.S.A.). To measure the nitrite concentration, the culture media of BV2 cells was changed to DMEM (low glucose) containing 1% FBS and the cells were then treated with TCA (1 or 3 μM). After 1 h, the cells were stimulated with 1 μg/mL of LPS for 24 h. The supernatant was then collected and mixed with an equal volume of Griess Reagent. The mixture was incubated for 10 min in the dark at room temperature. The absorbance was measured at 570 nm using an enzyme-linked immunosorbsent assay (ELISA) Reader. The nitrite concentration was calculated compared to a standard curve of sodium nitrite (NaNO₂) generated from several concentrations.

**Animals and Intracerebroventricular (i.c.v.) Injection of 6-OHDA** Animal experiments were carried out in accordance with the guidelines of the National Institute of Health (NIH) and the Korean Academy of Medical Sciences. Male adult ICR mice (6 weeks, 20–25 g) were housed at room temperature (22–23°C) under a 12 h light/12 h dark cycle with free access to food and water. To block the reuptake of norepinephrine, desipramine (25 mg/kg) was intraperitoneally injected into the right cerebral lateral ventricle (anterior (AP): −0.80 mm, lateral (ML): −1.20 mm, and vertical (DV): −3.60 mm by Bregma) at a rate of 0.5 μL/min using a 26-gauge Hamilton syringe. TCA (30 mg/kg, i.p.) dissolved in the phosphate-buffered saline (PBS) was administered 1 h before as well as 3, 7, 24 and 48 h after the 6-OHDA injection. Except for the sham control group (n=15), two groups of control mice (n=15) and 6-OHDA-injected mice (n=15) were treated with either TCA (TCA group) or an equal volume of PBS (6-OHDA group), respectively. A group of 6-OHDA-injected mice (n=15) was treated with TCA (TCA/6-OHDA group).

**Immunohistochemistry** The animals were anesthetized 7d after the 6-OHDA or vehicle injection and then perfused transcardially with 4% of paraformaldehyde in a 0.05 M of sodium phosphate buffer. The brains were removed, post-fixed, and cryosectioned. Immunohistochemistry was performed on free-floating cryocut sections (40 μm thick) that encompassed the entire striatum and SN. After incubation with H₂O₂ (3%) in 0.05% of PBS, incubation was performed with Triton X-100 (0.3%) and bovine serum albumin (BSA, 3%) in 0.1 M of PBS. After the entire staining protocol, the sections were then washed overnight at room temperature using anti-tyrosine hydroxylase (TH) antibody (1:1000, Santa Cruz Biotechnology, U.S.A.) to visualize dopaminergic neurons. The Vectastain ABC Kit (Vector Laboratories, U.S.A.) was used for the secondary antibody and then visualized with 3,3-diaminobenzidine (DAB) (40 mg of DAB, 0.045% of H₂O₂ in 100 mL of PBS) and mounted on gelatin-coated slides. To calculate the number of surviving neurons, the same region of the SN was selected. Tissue images were obtained using an optical microscope (Olympus, Japan) connected to a CCD camera to a PC monitor. The TH-positive neurons in the SN were counted. The neuronal cells were measured using an image analyzing system equipped with a computer-based CCD camera (software: Optimus, version 6.5, Media Cybernetics, MD, U.S.A.). Cell counting was performed by an analyzer blind to all treatments.

**Western Blot Analysis** For the Western blot analysis in the microglia, the cells were washed with PBS three times and lysed in the lysis buffer (20 mM of Tris pH 7.4, 150 mM of NaCl, Triton X-100 (1%), glycerol (10%), 2 mM of ethylene glycol–bis(beta-aminoethyl ether)–N,N,N',N'-tetraacetic acid (EGTA), 1 mM of phenylmethylsulfonyl fluoride (PMSF), 2 μg/mL of leupeptin, 1 μg/mL of pepstatin, and 1 mM of sodium orthovanadate). The cells were then scraped, transferred to a microtube and incubated for 30 min on ice, and finally centrifuged at 12000×g for 10 min at 4°C. Protein concentrations were determined based on the Bradford Reagent (Bio-Rad, U.S.A.) and 10 μg of total protein was loaded onto a 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After being transferred to a nitrocellulose membrane (Bio-Rad, U.S.A.), the membrane was blocked for 1 h at room temperature in a Tris-buffered saline (TBS) containing Tween-20 (0.1%) and skim milk (5%). The membrane was incubated overnight at room temperature with primary antibodies: mouse monoclonal anti-iNOS (1:5000, BD Pharmingen, U.S.A.) or mouse monoclonal anti-COX-2 (1:10000, Santa Cruz Biotechnology, U.S.A.) in TBS containing Tween-20 (0.1%). The primary antibodies were detected using a horseradish peroxidase (HRP)-conjugated secondary antibody (anti-mouse, 1:1000). The blots were finally visualized with an enhanced chemiluminescence (ECL) detection system (Amersham Biosciences, U.K.). The band intensity of the detected proteins was measured by densitometry. β-Actin was used as an internal control.

For the Western blot of the mice samples, the bilateral striatum and SN were dissected at 7d after the 6-OHDA or vehicle injection. The samples were homogenized in 400 μL of an ice-cold lysis buffer; 50 mM of Tris–HCl (pH 7.5), 150 mM of NaCl, Triton X-100 (1%), sodium deoxycholate (0.5%), SDS (0.1%), and sodium azide (0.02%). The samples (10 μg of proteins) were separated and then transferred to the membrane. The membrane was incubated with the primary antibodies: mouse monoclonal anti-TH (1:5000, Chemicon International Inc., U.S.A.), mouse monoclonal anti-iNOS (1:5000) or mouse monoclonal anti-COX-2 (1:10000).

**Statistical Analysis** The data was expressed as the mean±S.E.M. of three independent experiments or two through five subjects, and the statistical analyses were carried out by the one-way analysis of the variance (ANOVA). The significance was determined using the Newman–Keuls post-hoc test (Scheffe or Tukey HSD). A p<0.05 was considered to be significant in all analyses.

**RESULTS**

**Cytoprotective Effect of TCA** To estimate the protective effect of TCA in the BV2 cells against the damage induced by LPS, cell viability was measured using FDA/PI staining and an MTT assay (Fig. 1). The results showed that TCA has a protective effect on the BV2 microglial cells at both concentrations of TCA of 1 μM and 3 μM against LPS stimulation with no significant cytotoxicity at the concentrations of TCA itself
We next measured the levels of NO production and the expression levels of iNOS and COX-2 which are related to the inflammation. When the BV2 cells were stimulated with LPS alone, a strong induction of NO production was observed; the amount of NO production increased up to approximately 4.5 times compared to the control group (Fig. 2A). However, NO production was inhibited by the TCA treatment in a dose-dependent manner (Fig. 2A). In the absence of TCA treatment, LPS stimulation resulted in a marked induction of iNOS expression as compared to the control group. The TCA treatment, however, inhibited the LPS-induced iNOS expression (Fig. 2B). TCA treatment together with LPS also showed an inhibitory effect on COX-2 expression in the LPS-stimulated microglia (Fig. 2B). These observations show that LPS has an inflammation-stimulating function as an inflammatory stimulus and TCA has an efficacy to inhibit the inflammation induced by the LPS challenge.

**Neuroprotective Efficacy of TCA on the Dopaminergic Damages by 6-OHDA**

We next investigated the neuroprotective effect of TCA on a parkinsonism model using 6-OHDA which is the most widely used catecholaminergic neurotoxin to develop an experimental model of parkinsonism. A robust loss of TH-positive DA neurons was observed in the striatum and SN of the 6-OHDA-injected mice (Figs. 3C, G). Similar to the previous reports, these observations demonstrate that a 6-OHDA injection to the cerebral lateral ventricle induces the damage of dopaminergic nerve terminals in the striatum and the degeneration of nerve cell bodies in the SN (Figs. 3C, G). However, a significant increase of TH-positive DA neurons was observed in the mice injected with TCA as well as 6-OHDA, indicating the inhibitory effect of TCA on 6-OHDA-derived neurodegeneration (Figs. 3D, H). Seven days after the 6-OHDA injection, the total number of TH-positive DA neurons in the SN of the mice which had been injected with 6-OHDA only was reduced to 38.11 ± 11.01% when compared to the control, showing a cell death rate of about 62% (control SN: 100.00 ± 4.53%, TCA-treated SN: 98.09 ± 8.35%, TCA/6-OHDA-treated SN: 75.38 ± 9.06%) (Fig. 3I).

The expression patterns of TH were also examined both in the striatum and in the SN regions by Western blot analysis, which were very similar to the immunohistochemical observations (Figs. 4A, B). These observations indicate that TCA can effectively protect the TH-positive DA neurons against neurodegeneration which is induced by 6-OHDA neurotoxicity. In the *in vivo* experiment of this study, TCA was capable of suppressing the inductions of iNOS and COX-2 which dramatically increased at 7d after the 6-OHDA injection (lanes 3, 4 in Figs. 4A, B). TCA treatment...
only did not significantly affect the expression of TH, iNOS, and COX-2 in both regions when compared to the control (lanes 1, 2 in Figs. 4A, B).

DISCUSSION

This study was performed to show the efficacy of TCA to inhibit the progress of inflammation in the 6-OHDA-induced mouse parkinsonism model. We first needed to confirm how much TCA can inhibit the inflammatory reactions induced by LPS which is widely used to evoke inflammation. We have previously observed that the ethanol extract of CR has a potent anti-inflammatory activity. In addition, it was reported that TCA had a potent inhibitory effect on NO production and iNOS expression, while cinnamic acid and eugenol, which are components of Cinnamomum cassia, had little or no inhibitory effect on them. Thus, we next identified the level of NO production and the expression levels of iNOS and COX-2 which are related to the inflammation. It seems that TCA actually has a function to inhibit the inflammatory reactions induced by LPS in the cell culture study part of this study.

LPS stimulation itself is useful to induce an inflammatory response, which results in the dopaminergic cell degeneration both in vitro and in vivo. TCA (1 or 3 μM) showed a protective efficacy on the BV2 cells against LPS-induced cell toxicity with no significant cytotoxicity in the cells as shown in Fig. 1. Thus, the microglial cells activated by the LPS stimulation would release pro-inflammatory molecules such as interleukin (IL)-1β, tumor necrosis factor-α (TNF-α) and NO, which can be neurotoxic if they are overproduced. The NO derived from l-arginine by NO synthase (NOS) has been suggested to have a detrimental role in the inflammatory processes occurring in PD. Growing evidence suggests that iNOS is the most important enzyme involved in the regulation of inflammation. The observed results showed that TCA can inhibit NO production by suppressing iNOS expression in the microglia activated by the LPS challenge. Similar to this observation, it has been reported that TCA has a suppressing effect on NO production and NOS expression in RAW 264.7 cells. The activated microglia by the LPS stimulation showed up-regulated levels of COX-2, a key enzyme responsible for the synthesis of inflammation-related prostaglandins, which can trigger direct toxicity in the dopamine-containing neurons. COX-2 is being strongly considered to play a crucial role in the neurodegenerative processes and in the intense inflammatory responses followed by neuronal death eventually in an over-threshold condition to cells. Indeed, the suppression of COX-2 alone or both COX-1 and COX-2 by selective and non-selective non-steroidal anti-inflammatory drugs (NSAIDs) in neural injury models has been observed to exert a neuroprotective effect. A previous study showed that CR, which contains TCA as a main component, suppresses COX-2 expression in the LPS-stimulated RAW264.7 cells. It was also reported that TCA can inhibit COX-2 expression in vitro. Correlating with these reports, this study showed that TCA may have an anti-inflammatory activity because it was observed that TCA may inhibit the inflammatory events in the BV2 microglia by suppressing the expressions of iNOS.
We next investigated the neuroprotective efficacy of TCA on a 6-OHDA-induced parkinsonism model. 6-OHDA is the most widely used catecholaminergic neurotoxin to develop an experimental model of parkinsonism.\(^2\) 6-OHDA causes nigrostriatal DA neuronal degeneration when it is directly injected into the medial forebrain bundle, striatum or SN.\(^2\) The i.c.v. injection of 6-OHDA induces a dopaminergic dysfunction in mice as well.\(^2\) 6-OHDA has been reported to cause a more severe depletion of DA than 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in a previous study.\(^2\) Hence, it seems that 6-OHDA is the most useful neurotoxin to evaluate the neuroprotective efficacy of TCA. TH catalyzes the conversion of \(\text{L-tyrosine}\) to \(\text{L-dihydroxyphenylalanine (L-DOPA)}\), which is the rate-limiting first step in the biosynthesis of DA.\(^2\) Thus, TH immunoreactivity has been used to evaluate the 6-OHDA-induced neurotoxicity in the striatum and SN areas.

Although 6-OHDA leads to the apoptosis of nigrostriatal dopaminergic cells, a report showed that the toxic effects of 6-OHDA are partially mediated through the activation of the microglia.\(^2\) Direct administration of 6-OHDA into the striatum of mice activates the microglia in the SN and increases the number of activated microglia in the SN, causing the subsequent increase in the activated microglia after one week.\(^2\) Some researchers have investigated the microglial response to progressive dopaminergic neurodegeneration and concluded that neuroinflammation is a significant channel of...
the 6-OHDA-induced neurodegenerative process. In addition, high levels of neuronal and inducible NOSs (nNOS and iNOS) were observed in the SN of PD patients and parkinsonism model. These reports suggest that iNOS may play a role in the pathogenesis of PD which is related to CNS inflammation. It needs to be noted that the inflammatory genes coding iNOS and COX-2 have been found to be up-regulated in the amoeboid microglia in the PD patients. In the in vivo experiment of this study TCA was capable of suppressing the induction levels of iNOS and COX-2 which were highly increased at seven days after the 6-OHDA injection as shown in the Fig. 4. These results show that the expression of iNOS and COX-2 can be effectively inhibited by TCA treatment and TCA may inhibit the activation of microglia in the 6-OHDA-induced nigrostriatal injuries.

DA is released not only from the axonal terminus of the nigrostriatal DA neurons in the striatum but also from the dendrites of DA neurons’ nuclei in the SN. 3,4-Dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) are two important intermediates in the metabolic processes of DA and directly reflect the change of DA contents in the CNS. In addition, many studies reported that the decrease of DA and its metabolites was observed in the course of the disease in the PD patients and the animal models of parkinsonism similar to this study. Even accounting for some variation according to the mice and not significant, it seemed that TCA has an ameliorative efficacy to weakly inhibit the reduction of DA and its metabolites induced by the 6-OHDA challenge (data not shown). We have applied two behavioral tests (rotational turn test and rotarod test) to this study to evaluate the behavioral amelioration of TCA-treated mice against 6-OHDA, but failed to attain a significant observation (data not shown). This failure may reflect a relatively large variation of S.E.M. in the content of DA and its metabolites in the striatum, even with a molecular significance in the expression of TH, iNOS, and COX-2 between the TCA-treated parkinsonism mice and 6-OHDA-treated only.

The anti-inflammatory effect of TCA was observed in both LPS-induced microglia and 6-OHDA-injected mice in this study. It has been reported that inflammation may induce the dopaminergic neurodegeneration and that anti-inflammatory drugs may provide the neuroprotective properties by inhibiting the inflammation. Thus, we hypothesized that the preventive effects of TCA on the dopaminergic dysfunction induced by 6-OHDA may be associated with the anti-inflammatory effects of TCA. Our current results suggest that TCA has a neuroprotective efficacy on nigrostriatal damage in the 6-OHDA parkinsonism model and may inhibit inflammatory responses in the nigrostriatal system. TCA is known to be an active component which can be derived from the bark of Cinnamomum cassia. We previously observed that CR (young twigs of Cinnamomum cassia) especially can inhibit the activation of many inflammatory genes to suppress the inflammatory responses following the microglial activation in the nigrostriatal system by inhibiting the activation of many inflammatory genes or through the LRP1. On the other hand, TCA may directly have a protective efficacy on the 6-OHDA-induced degeneration of TH-positive dopaminergic neurons as shown in the immunohistochemical stainings in Fig. 3, or indirectly via microglia and astrocytes in the nigrostriatal regions. It is well known that nuclear factor-kB (NF-kB) is a transcription factor for iNOS and COX-2. Thus, NF-κB activation may be influenced by 6-OHDA and TCA, which indicates that NF-kB may be activated in the nigrostriatal system by 6-OHDA, inducing inflammatory reaction including the up-regulation of iNOS and COX-2, but that TCA may inhibit the NF-κB activation, inhibiting the inflammatory reaction, thereby maintaining the basic expression levels of iNOS and COX-2. Further researches are required to investigate the underlying mechanism of the neuroprotective efficacy of TCA, which might result in the development of safe and effective neuroprotective drugs based on TCA for neurodegenerative disorders.

CONCLUSION

In the present study, it was shown that TCA has the anti-inflammatory efficacy to inhibit inflammation induced by both LPS-stimulated microglial activation and 6-OHDA-induced dopaminergic damages.

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REFERENCES


50) Chen H, Jing FC, Li CL, Tu PP, Zheng QS, Wang ZH. Echinacosaic
prevents the striatal extracellular levels of monoamine neurotransmitters from diminution in 6-hydroxydopamine lesion rats. *J. Ethnopharmacol.*, 114, 285–289 (2007).


