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Anti-inflammatory effects of catechols in lipopolysaccharide-stimulated microglia cells: Inhibition of microglial neurotoxicity

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ABSTRACT

Microglial activation plays a pivotal role in the pathogenesis of neurodegenerative diseases by producing various proinflammatory cytokines and nitric oxide (NO). In the present study, the anti-inflammatory and subsequent neuroprotective effects of catechol and its derivatives including 3-methylcatechol, 4-methylcatechol, and 4-*tert*-butylcatechol were investigated in microglia and neuroblastoma cells in culture. The four catechol compounds showed anti-inflammatory effects with different potency. The catechols significantly decreased lipopolysaccharide (LPS)-induced NO and tumor necrosis factor (TNF)-α production in BV-2 microglia cells. The catechols also inhibited the expression of inducible nitric oxide synthase (iNOS) and TNF-α at mRNA or protein levels in the LPS-stimulated BV-2 cells. In addition, the catechols inhibited LPS-induced nuclear translocation of p65 subunit of nuclear factor (NF)-κB, IκB degradation, and phosphorylation of p38 mitogen-activated protein kinase (MAPK) in BV-2 cells. Moreover, the catechols attenuated the cytotoxicity of LPS-stimulated BV-2 microglia toward co-cultured rat B35 neuroblastoma cells. The catechols, however, did not protect B35 cells against H_2O_2 toxicity, indicating that the compounds exerted the neuroprotective effect by inhibiting the inflammatory activation of microglia in the co-culture. The anti-inflammatory and neuroprotective properties of the catechols in cultured microglia and neuroblastoma cells suggest a therapeutic potential of these compounds for the treatment of neurodegenerative diseases that are associated with an excessive microglial activation.

1. Introduction

Inflammation is the first response of an organ's immune system to the pathogenesis. Previous studies have demonstrated that inflammation is actively involved in the pathogenesis of neurodegenerative diseases including Alzheimer's disease, Parkinson's disease multiple sclerosis, and HIV-associated dementia (Banati et al., 1998; Block et al., 2007; McGeer et al., 1988; Raine, 1994). Microglia, which are responsible for the innate immunity in the brain, are considered to be the major cell type responsible for inflammation-mediated neurotoxicity (Liu and Hong, 2003; McGeer et al., 2001). Activation of microglia is often observed in neuronal injuries and is also induced after stimulation with lipopolysaccharide (LPS), interferon (IFN)-γ or β-amyloid *in vitro* (Giulian et al., 1994; Illes et al., 1996). The activated microglia has the capability of releasing a variety of soluble proinflammatory factors such as tumor necrosis factor (TNF)-α, interleukin (IL)-1 and IL-6 and free radicals like nitric oxide (NO) and superoxide anion (Liu and Hong, 2003; McGeer et al., 2001). In the brain, microglia degrade and eliminate apoptotic neuronal cells (Stolzing and Grune, 2004). However, several studies have reported that microglia also promote the death of Purkinje cells in the developing mouse cerebellum. Selective elimination of microglia can strongly inhibit Purkinje cell death, which suggests that microglia may exacerbate neuronal cell death under neurodegenerative conditions (Marin-Teva et al., 2004). Moreover, it has been demonstrated that activated microglia play an active role as a possible cause of neurodegenerative diseases (Block et al., 2007; Gonzalez-Scarano and Baltuch, 1999; Lim et al., 2001; McGeer et al., 1988). Therefore, the modulation of microglia activation and neuroinflammation may be an effective therapeutic approach against neurodegenerative diseases.

Catechol was first isolated in 1839 by H. Reinsch by distilling catechin (the juice of *Mimosa catechu*); its sulfonic acid is present in the urine of horse and man (Von Euler et al., 1955). Urushiols and catecholamines are naturally existing organic compounds which have a catechol skeleton structure. Urushiols are the skin-irritating poisons found in plants like poison ivy, etc. Catecholamines are biochemically significant hormones/neurotransmitters, where the phenyl group has a catechol skeleton structure. Small amounts of catechol occur naturally in fruits and vegetables, along with the enzyme polyphenol oxidase. Upon exposure to air (as when a potato or apple is cut), the colorless catechol oxidizes to reddish-brown benzoquinone, which is responsible for the browning of cut fruit and vegetables. A catechol skeleton structure is also found in the polyphenolic compounds such as quercetin and catechins, which have been found to exhibit a wide range of biological activities including antioxidant, anti-inflammatory and neuroprotective effects (Chen et al., 2005; Dias et al., 2005; Li et al., 2004). It was suggested that polyphenols are rapidly metabolized into catechols in the digestive organs after ingestion (Graefe

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et al., 1999; Hollman and Katan, 1999; Wiseman, 1999). Therefore, many studies have compared the biological activities of catechols with the parent compounds. For instance, the antioxidant activities of quercetin and its possible metabolite 4-methylcatechol have been investigated (Glasser et al., 2002). Catechol, 3-methylcatechol, and 4-*tert*-buthylcatechol are possible metabolites of other polyphonic compounds. Several studies have reported that 4-metylcatechol can stimulate the synthesis of brain-derived neurotrophic factor and nerve growth factor in both neurons and astrocytes (Furukawa and Furukawa, 1990; Nitta et al., 1999). On the other hand, a number of studies have demonstrated that the parent compounds (catechins and quercetin) have an anti-inflammatory effect on LPS-stimulated macrophages (Chen et al., 2005; Li et al., 2004). Alanko et al. (1999) have reported that 3-methylcatechol and 4-methylcatechol inhibited prostaglandin E2 synthesis in A23187-stimulated human polymorphonuclear leukocytes.

Although the anti-inflammatory effect of polyphenolic compounds has been reported, the possible anti-inflammatory effect of their catechol metabolites has not been investigated. Here, we determined the effect of catechol and its derivatives such as 3-methylcatechol, 4-methylcatechol, and 4-*tert*-butylcatechol on the inflammatory activation and neurotoxicity of BV-2 microglia cells. It was found that these compounds inhibited the production of NO and TNF-α, and suppressed NF-κB and p38 mitogen-activated protein kinase (MAPK) activation in LPS-stimulated BV-2 microglia cells. In addition, these compounds showed neuroprotective effects by attenuating microglial neurotoxicity in a microglia-neuron co-culture system.

2. Materials and methods

2.1. Reagents and cell culture

Bacterial LPS (*E. coli* serotype 055:B5), 3-methylcatechol, 4-methylcatechol, 4-*tert*-butylcatechol, catechol, *N*G-monomethyl-L-arginine (L-NMMA) and SB203580 (4-(4-fluorophenyl)-2-(4-methylsulphinylphenyl)-5-(4-pyridyl) imidazole) were purchased from Sigma-Aldrich (St. Louis, MO). A BV-2 murine microglia cell line was grown and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% heat-inactivated fetal bovine serum (FBS), gentamicin (50 μg/ml) at 37 °C, 5% CO_2. A B35 rat neuroblastoma cell line was grown and maintained in DMEM supplemented with 10% heat-inactivated FBS, penicillin (10 U/ml) and streptomycin (10 μg/ml) at 37 °C, 5% CO_2. For co-culture experiment, BV-2 microglia cells were seeded in triplicate at the concentration of 1.5×10^4 cells/well in 96-well plates. BV-2 microglia cells were pretreated with catechols of 2 μg/ml for 30 min. Then, culture supernatants were discarded and 0.5 μg/ml of LPS was added together with B35 rat neuroblastoma cells stably expressing EGFP (3×10^4 cells/well), which was followed by the co-culture for 24 h. Afterwards, the EGFP-positive cells were counted under fluorescence microscope (Olympus IX 70, Tokyo, Japan). Images of three random fields per well were captured and analyzed by the MetaMorph imaging system (Universal Imaging Corp, West Chester, PA). LPS alone did not affect B35 neuroblastoma cell viability (data not shown).

2.2. Cell viability test

Cell viability was determined by 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The BV-2 microglia cells were seeded in triplicate at the density of 5×10^4 cells/well on 96-well plate. BV-2 microglia cells were treated with various compounds for 24 h. MTT was added to each well, and the cells were incubated for 4 h at 37 °C. After culture media were discarded, dimethyl sulfoxide (DMSO) was added to dissolve the formazan dye. The optical density was measured at 540 nm.

2.3. Nitrite quantification

NO secreted in microglial culture supernatants was measured by Griess reagent as described (Lee and Suk, 2007). After BV-2 microglia cells were treated with activating agents on 96-well plates, NO_2^- concentration in culture supernatants was measured to assess NO production in microglial cells. Fifty microliters of sample aliquots were mixed with 50 μl of Griess reagent (1% sulfanilamide/0.1% naphthylethylene diamine dihydrochloride/2% phosphoric acid) on a 96-well plate and incubated at 25 °C for 10 min. The absorbance at 550 nm was measured on a microplate reader. $NaNO_2$ was used as the standard to calculate NO_2^- concentrations.

2.4. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated using TRIzol reagent (Molecular Research Center Inc., Cincinnati, OH) according to the manufacturer's instruction. Reverse transcription was carried out using Moloney murine leukemia virus (M-MLV) and oligo (dT) primer. PCR amplification using primer sets specific for inducible nitric oxide synthase (iNOS), TNF-α, or β-actin was carried out at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min, and repeated over 23 cycles, followed by incubation at 72 °C for 7 min. Nucleotide sequences of the primers were based on published cDNA sequences of mouse iNOS, TNF-α, or β-actin: iNOS forward, CCC TTC CGA AGT TTC TGG CAG CAG C; iNOS reverse, GGC TGT CAG AGC CTC GTG GCT TTG G; TNF-α forward, CAT CTT CTC AAA ATT CGA GTG ACA A; TNF-α reverse, ACT TGG GCA GAT TGA CCT CAG; β-actin forward, ATC CTG AAA GAC CTC TAT GC; β-actin reverse, AAC GCA GCT CAG TAA CAG TC. The β-actin was used as an internal control to evaluate relative expression of iNOS and TNF-α.

2.5. Immunofluorescence assay

For the detection of intracellular location of p65 subunit of NF-κB, BV-2 microglia cells (1×10^5 cells/well in 24-well plates) were cultured on sterile cover slips in 24-well plates and treated with compounds and LPS. At 60 min after the LPS treatment, the cells were fixed with methanol for 20 min at −20 °C and washed with PBS for 5 min. The fixed cells were then permeabilized with 0.5% Triton X-100 in PBS for 1 h at room temperature, washed with 0.05% Tween-20 in PBS for 10 min and 0.05% Tween-20/1% BSA in PBS for 5 min. The permeabilized cells were then treated with 1 μg/ml of monoclonal mouse anti-human NF-κB p65 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for 60 min at room temperature, washed with 0.05% Tween-20/1% BSA in PBS for 5 min. Cells were then incubated in a 1:2000 dilution of Alexa Fluor 488-labeled goat anti-mouse antibody (Molecular Probes Inc., Eugene, OR) for 60 min at room temperature, and washed with 0.05% Tween-20 in PBS for 5 min and PBS for 5 min. Cells were then stained with 0.5 μg/ml of Hoechst staining solution for 20 min at 37 °C and then washed. Finally, the cover slips with cells were dried at 37 °C in an oven for 45 min and mounted in a 1:1 mixture of xylene and malinol. More than 50 cells per field were counted under a fluorescence microscope.

2.6. Western blot analysis

Cells were lysed in a triple-detergent lysis buffer [50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.02% sodium azide, 0.1% sodium dodecyl sulfate (SDS), 1% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride]. Protein concentration in cell lysates was determined using a protein assay kit (Bio-Rad, Hercules, CA). An equal amount of protein from each sample was separated by SDS-polyacrylamide gel electrophoresis and transferred to Hybond ECL nitrocellulose membranes (Amersham Biosciences, Piscataway, NJ).

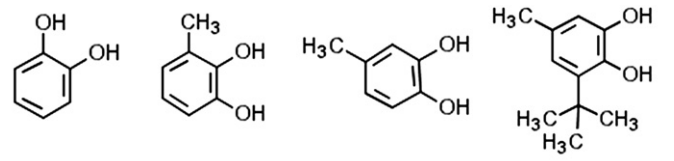

Fig. 1. The chemical structure of the catechols used in this study.

The membranes were blocked with 5% skim milk and sequentially incubated with primary antibodies [polyclonal rabbit anti-mouse iNOS (BD Biosciences, Franklin Lakes, NJ), polyclonal rabbit anti-human IκB-α (Santa Cruz Biotechnology), polyclonal rabbit anti-human phospho-p38 MAPK (Cell Signaling Technology Inc., Beverly, MA), monoclonal anti-α-tubulin clone B-5-1-2 mouse ascites fluid (Sigma)] and horseradish peroxidase-conjugated secondary antibodies (anti-rabbit or anti-mouse IgG; Amersham Biosciences) followed by enhanced chemiluminescence detection (Amersham Biosciences).

2.7. Enzyme-linked immunosorbent assay (ELISA)

TNF-α secreted in microglial culture supernatants was measured as described (Park et al., 2002) by specific ELISA using rat monoclonal

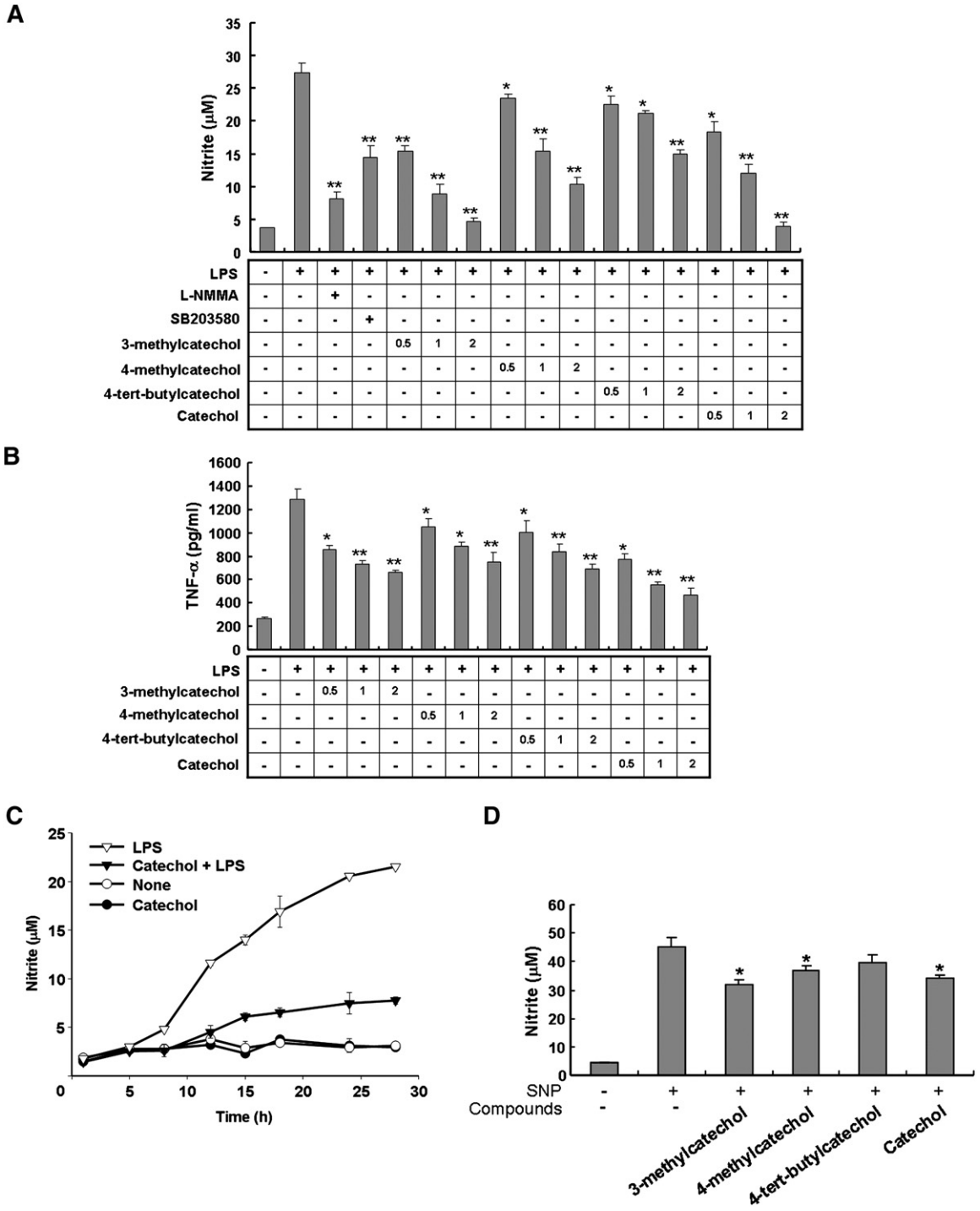

Fig. 2. Effect of the catechols on NO (A, C, D) and TNF-α (B) production in LPS-stimulated BV-2 microglia. The BV-2 microglia cells were seeded in triplicate at the density of 5×10^4 cells/well on 96-well plate. BV-2 microglia cells were treated with 0.1 μg/ml of LPS in the presence or absence of indicated concentrations of catechols (0.5–2 μg/ml) or L-NMMA (500 μM) or SB203580 (10 μM) for 24 h (A, B) or for the indicated time period with 2 μg/ml of catechol (C). The amounts of nitrite (A, C) or TNF-α (B) in the supernatants were measured using Griess reagent or ELISA, respectively. (D) BV-2 microglia cells were co-incubated with 100 μM of SNP in the presence or absence of catechols (2 μg/ml) for 24 h without LPS stimulation. The amounts of nitrite in the supernatants were measured using Griess reagent. The data were expressed as the mean ± S.D. ($n=3$), and are representative of results obtained from three independent experiments. *$P<0.05$, **$P<0.01$; significantly different from the value in cells treated with LPS or SNP alone.

Table 1
The IC50 values of the catechol compounds for inhibiting LPS-induced NO production in BV-2 microglia

Compounds	IC_{50}	
	(μg/ml)	(μM)
Catechol	0.61 ± 0.12	5.56 ± 1.07
3-methylcatechol	0.45 ± 0.15	3.63 ± 1.17
4-methylcatechol	1.07 ± 0.21	8.64 ± 1.71
4-*tert*-butylcatechol	2.08 ± 0.23	12.56 ± 1.38

Data were presented as 50% inhibitory concentration (IC_{50}). Values are the mean ± S.D. of five independent experiments.

anti-mouse TNF-α antibody as capture antibody and goat biotinylated polyclonal anti-mouse TNF-α antibody as detection antibody (ELISA development reagents; R&D Systems, Minneapolis, MN). The biotinylated anti-TNF-α antibody was detected by sequential incubation with streptavidin-horseradish peroxidase conjugate and chromogenic substrates.

2.8. Statistical analysis

Results were expressed as mean ± S.D. The data were analyzed by one-way ANOVA following the Student Newman Keul's post hoc analysis, using SPSS program (version 12.0). A value of $P<0.05$ was considered statistically significant.

3. Results

3.1. Catechols inhibited the NO and TNF-α production

In order to investigate the anti-inflammatory effect of catechols in microglia cells, catechol and its derivatives (3-methylcatechol, 4-

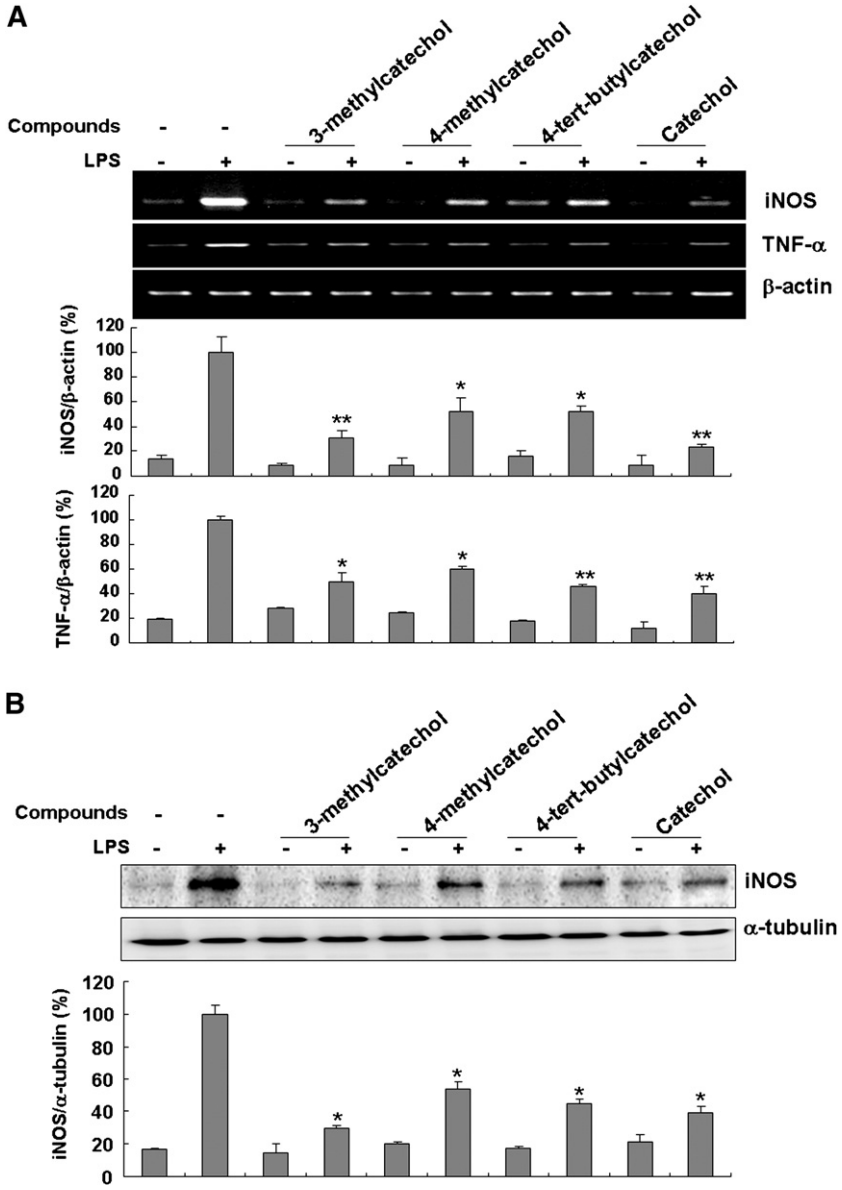

Fig. 3. Effect of the catechols on iNOS and TNF-α gene expression in LPS-stimulated BV-2 microglia cells. BV-2 microglia cells were pretreated with the catechols (2 μg/ml) for 30 min and then treated with LPS (0.1 μg/ml). (A) The total RNA was isolated at 6 h after the LPS treatment. The iNOS and TNF-α mRNA levels were determined by RT-PCR (*upper*), and then subjected to densitometric quantification (*lower*). Levels of iNOS and TNF-α were normalized to β-actin levels and expressed as a relative change in comparison to the LPS treatment, which was set to 100%. (B) After 12 h of LPS stimulation, the cell lysates (40 μg) were separated by SDS-PAGE, transferred to a nitrocellulose membrane and probed with anti-iNOS antibody (*upper*). The α-tubulin was used as an internal control. Quantification of iNOS protein expression was performed by densitometric analysis (*lower*). The values were expressed as a percentage of maximal band intensity in the culture treated with LPS alone, which was set to 100%. The data were expressed as the mean ± S.D. ($n=3$), and are representative of results obtained from three independent experiments. *$P<0.05$, **$P<0.01$; significantly different from the value in cells treated with LPS only.

methylcatechol, and 4-*tert*-butylcatechol) (Fig. 1) were tested for their effects on NO and TNF-α production in LPS-stimulated BV-2 microglia cells. After stimulation of BV-2 microglia cells with LPS (0.1 μg/ml) for 24 h, NO concentration was significantly increased in culture medium. When cells were treated with L-NMMA (iNOS inhibitor) or SB203580 (p38 MAPK inhibitor) the LPS-induced production of NO was significantly decreased (Fig. 2A). LPS-elicited NO and TNF-α production was also decreased by catechols in a dose-dependent manner (Fig. 2A, B). We also tested the effects of the catechols at an equal molar concentration due to there being a slight difference in the molecular weight among the compounds. A similar result of NO inhibition was obtained (data not shown). As shown in Fig. 2C, NO production was markedly increased between 12 h and 28 h after LPS treatment, and this was inhibited by treatment with 2 μg/ml of catechol. A similar result of time-dependent NO inhibition was obtained for other catechol compounds (data not shown). After 24 h of LPS stimulation, catechols showed a concentration-dependent inhibition of NO production (Fig. 2A). The potency of suppression of NO production was arranged as follows based on the 50% inhibitory concentration (IC_{50}): 3-methylcatechol>catechol>4-methylcatechol>4-*tert*-butylcatechol (Table 1). As catechols can be auto-oxidized to quinones and superoxide, which would rapidly react with NO, we examined whether catechols can scavenge the reactive nitrogen species by co-incubating the catechols with a NO donor sodium nitroprusside (SNP) in BV-2 microglia cells. As shown in Fig. 2D, the co-incubation of the catechols with SNP for 24 h partly suppressed the NO production compared with SNP treatment alone. The results indicated that catechols may inhibit the LPS-induced NO production through dual mechanisms: i) catechols may reduce NO production by inhibiting the LPS signaling in microglia; and ii) catechols may directly scavenge NO. In order to exclude the possibility that the decrease of NO or TNF-α production was due to the cytotoxicity of the compounds, the MTT assay was performed. The compounds did not have a significant cytotoxicity toward BV-2 microglia cells in the presence or absence of LPS *(data not shown).*

3.2. Catechols inhibited the expression of iNOS and TNF-α genes

To examine the effect of catechols on iNOS and TNF-α gene expression at the transcriptional level, we determined the levels of iNOS and TNF-α mRNA by RT-PCR analysis in the LPS-stimulated BV-2 microglia cells. The 3-methylcatechol, 4-methylcatechol, 4-*tert*-butylcatechol and catechol at 2 μg/ml inhibited the LPS-induced expression of iNOS and TNF-α, whereas the compounds alone did not result in any significant change of gene expression (Fig. 3A). The effect of the catechols on iNOS gene expression was further assessed at the protein levels by Western blot analysis. As shown in Fig. 3B, the catechols inhibited the LPS-induced iNOS protein levels to varying extents. The compounds alone did not significantly influence iNOS expression.

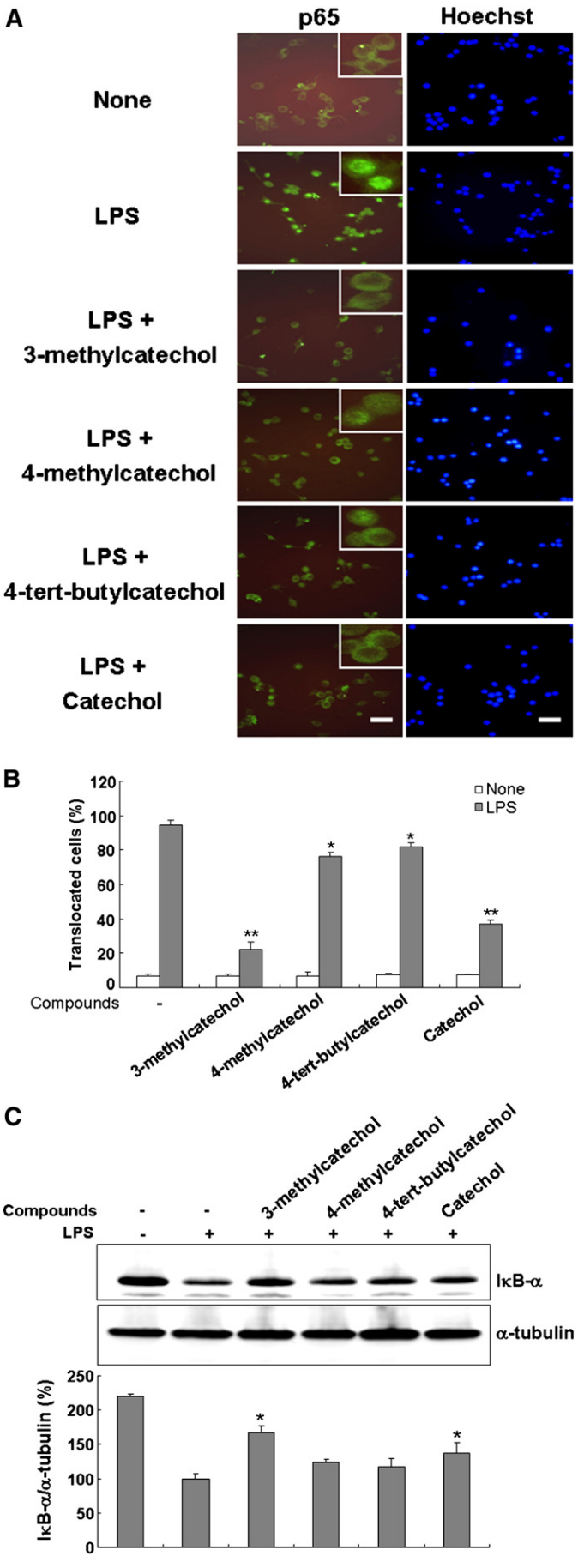

Fig. 4. Blockade of NF-κB activation by the catechols. The BV-2 microglia cells were seeded in triplicate at the density of 1×10^5 cells/well on 24-well plate. BV-2 microglia cells were stimulated with 0.1 μg/ml of LPS in the absence or presence of the catechols (2 μg/ml) that had been added 30 min before the stimulation. (A) At 1 h after the LPS addition, subcellular location of NF-κB p65 subunit was determined by immunofluorescence assay. The p65 protein was detected using anti-p65 antibody conjugated with fluorescein isothiocyanate (FITC), and nuclei were visualized by Hoechst staining (magnification, 200×). Representative images of cells are shown (higher magnification in inset). Scale bar = 50 μm. (B) The number of cells with p65 nuclear translocation was determined and the percentage of cells with p65 translocation was calculated: more than 50 cells per field were counted. (C) Total cell lysates obtained 30 min after the LPS stimulation were subjected to Western blotting to assess the levels of IκB-α proteins (*upper*). Quantification of IκB-α degradation was performed by densitometric analysis (*lower*). Detection of α-tubulin was done to confirm the equal loading of the samples. The values were expressed as a percentage of the band intensity in the culture treated with LPS alone, which was set to 100%. The data were expressed as the mean ± S.D. ($n=3$). Results are representative of more than three independent experiments. *$P<0.05$, **$P<0.01$; significantly different from the value in cells treated with LPS alone.

3.3. Catechols attenuated the LPS-induced NF-κB and p38 MAPK activation

The expression of iNOS and numerous proinflammatory genes in microglia requires NF-κB and p38 MAPK activation. The processes of NF-κB activation include IκB degradation and subsequent nuclear translocation of p65 subunit of NF-κB. Therefore, we determined whether the catechols inhibited p65 nuclear translocation. LPS induced the translocation of p65 into nucleus within 60 min of stimulation, which was almost completely inhibited by the presence of 3-methylcatechol and catechol at 2 μg/ml, as determined by immunofluorescence assay and subsequent enumeration of the cells with p65 translocation (Fig. 4A, B). The nuclear translocation of p65 was partially inhibited in the presence of 4-methylcatechol and 4-*tert*-butylcatechol. The compounds alone did not affect the nuclear translocation of p65 (Fig. 4B). The catechol and 3-methylcatechol also blocked degradation of IκB at 30 min after the LPS treatment (Fig. 4C). The p38 MAPK pathways are involved in the activation of microglia by regulating the synthesis and release of proinflammatory mediators (Koistinaho and Koistinaho, 2002). We investigated the effect of the catechols on LPS-induced activation of p38 MAPK. After 30 min stimulation with LPS, phosphoryation of p38 was strongly induced. The catechols, especially 3-methylcatechol and catechol, markedly inhibited the LPS-induced phosphorylation of p38 MAPK (Fig. 5). The compounds alone did not affect the phosphorylation of p38 MAPK (data not shown).

3.4. Catechols inhibited microglial neurotoxicity in a microglia/neuron co-culture model

To investigate the potential neuroprotective effect of catechols *in vitro*, microglia/neuron co-culture model was employed. As neuronal cell death is caused by proinflammatory mediators secreted from activated microglia (Stoll and Jander, 1999; Streit et al., 1999), inhibition of microglial activation may protect co-cultured neurons against the cytotoxic effect of activated microglia. We tested this possibility using a co-culture of B35 neuroblastoma cells with LPS-activated BV-2 microglia cells. BV-2 microglia cells were activated with LPS with or without pretreatment with the catechols, and then co-cultured with B35 neuroblastoma cells (see Fig. 6A for the co-culture scheme). The viability of B35 cells was measured by counting the EGFP expressing cells in the co-culture, because B35 cells stably expressed EGFP. This co-culture system has been successfully used for the determination of microglial neurotoxicity (Kim et al., 2007). The 3-methylcatechol, 4-methylcatechol, 4-*tert*-butylcatechol, and catechol inhibited B35 cell death induced by activated BV-2 microglia cells by 86, 50, 47 and 79%, respectively (Fig. 6B). The catechols, however, did not protect B35 cells against reactive oxygen species (ROS) toxicity (Fig. 6C). The treatment of B35 cells with H_2O_2 induced the cell death, which was not significantly influenced by the catechols. The results indicate that the catechols may be neuroprotective by inhibiting proinflammatory activation of microglia.

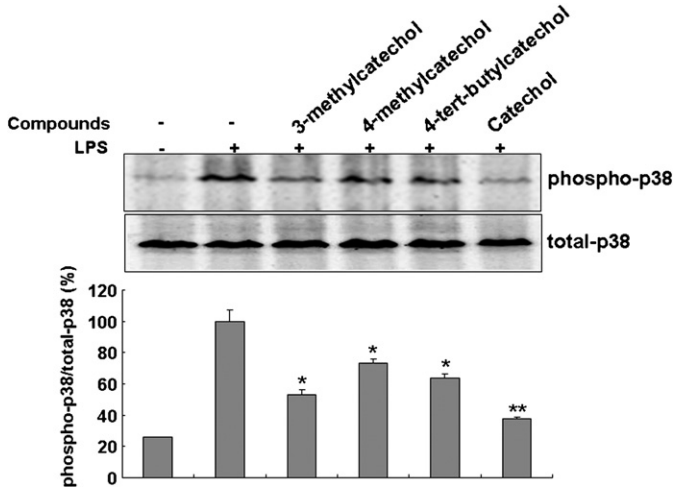

Fig. 5. The catechols suppressed LPS-induced phosphorylation of p38 MAPK. BV-2 microglia cells were stimulated with 0.1 μg/ml LPS in the absence or presence of catechols (2 μg/ml) that had been added 30 min before the activation. Total cell lysates obtained 30 min after the activation were subjected to Western blotting to assess the levels of phosphorylated p38 MAPK protein (phospho-p38) (*upper*), followed by densitometric quantification (*lower*). Detection of total p38 MAPK was also done. The values were expressed as a percentage of maximal band intensity in the culture treated with LPS alone, which was set to 100%. The data were expressed as the mean±S.D. (n=3), and are representative of results obtained from three independent experiments. *P<0.05, **P<0.01; significantly different from the value in cells treated with LPS only.

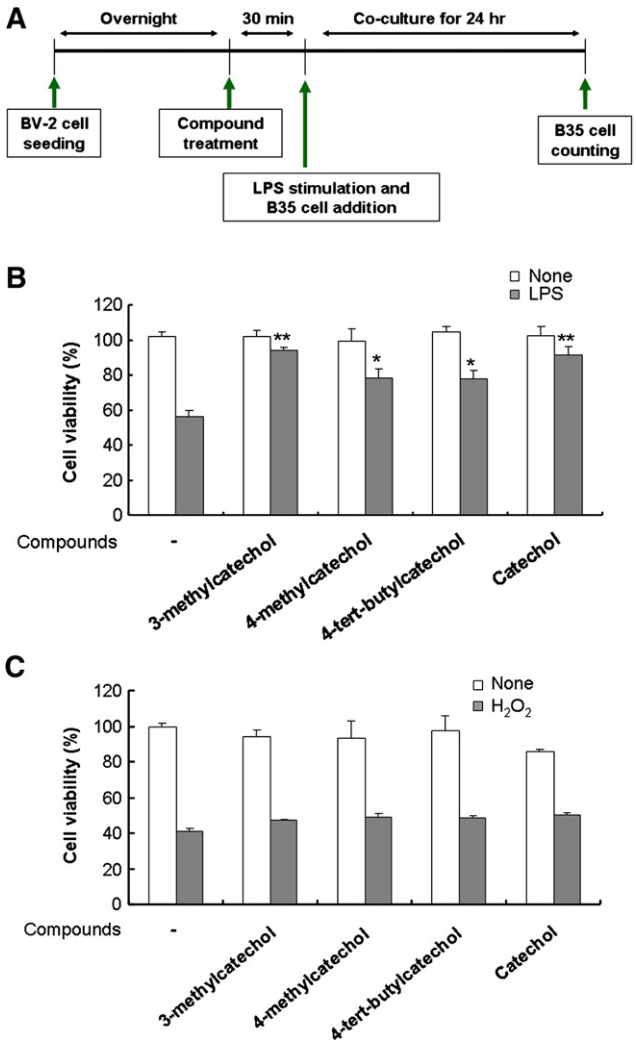

Fig. 6. Effect of the catechols on B35 neuroblastoma cell viability in the co-culture with BV-2 microglia cells. The BV-2 microglia cells were seeded in triplicate at the density of 1.5×10^4 cells/well on 96-well plate. BV-2 microglia cells were pretreated with the catechols (2 μg/ml) for 30 min. Culture supernatants were discarded and stimulated with 0.5 μg/ml of LPS. At the same time, rat B35 neuroblastoma cells (3×10^4 cells/well) stably expressing EGFP were added onto BV-2 cells, and then co-cultured for 24 h (A). (B) The number of fluorescent cells in three randomly chosen microscopic fields per well was determined, and the data were expressed as the mean±S.D. (n=3). Data are representative of results obtained from three independent experiments. *P<0.05, **P<0.01; significantly different from the value in cells treated with LPS in the absence of the catechols. (C) Alternatively, B35 cells were treated with H_2O_2 (0.5 mM) in the presence or absence of the catechols for 24 h. Cell viability was determined by MTT assay and the results were expressed as percentage of surviving cells over control cells (mean±S.D.; n=3). The data are representative of results obtained from three independent experiments.

4. Discussion

Activated microglia produce various proinflammatory cytokines and free radicals. Several lines of evidence have indicated that the expression of iNOS, a key enzyme for NO production, is upregulated in the activated microglia (McGeer et al., 1993). Additionally, proinflammatory cytokines such as TNF-α, IL-1β, IL-6 have an important role in the process of neuroinflammatory diseases. In this study, we demonstrated that catechols, metabolites of polyphenolic compounds, inhibited inflammatory activation of microglia in culture. Four types of catechols (3-methylcatechol, 4-methylcatechol, 4-*tert*-butylcatechol, and catechol) reduced the LPS-stimulated NO production in BV-2 microglia cells in a dose- and time-dependent manner. The catechols also suppressed iNOS gene expression at mRNA and protein levels. The NOS inhibitor, NMMA, significantly reduced NO production in the LPS-stimulated BV-2 microglia. Previous studies have demonstrated that specific iNOS inhibitor, aminoguanidine, reduced NO and ROS production in LPS/IFN-γ-simulated microglia (Hemmer et al., 2001; Reis et al., 2006). It has been also reported that another specific iNOS inhibitor, 1400 W, reduced microglia activation-mediated neuronal cell death in a neuron/microglia co-culture system (Mander and Brown, 2005). Based on the previous reports and the present results, it is clear that the inhibition of iNOS is one of the anti-inflammatory mechanisms of catechols in BV-2 microglia cells (Figs. 2 and 3). In addition, catechols were able to scavenge NO (Fig. 2D), providing an additional mechanism of NO inhibition. TNF-α is another main inflammatory mediator in neuroinflammatory diseases. In this study, we found that LPS induced TNF-α at mRNA as well as protein levels, whereas catechols significantly inhibited TNF-α expression at both levels. Consistent with the present studies, it was previously reported that catechol (100 μM) and other phenolic antioxidants completely blocked the TNF-α gene expression in LPS-stimulated RAW 264.7 macrophages (Ma and Kinneer, 2002). Furthermore, the compounds were neuroprotective based on the co-culture assay, in which the catechols partly prevented B35 neuroblastoma cell death by inhibiting the microglial activation. Catechols contain a large class of substances of natural or synthetic origin, and are known to exhibit numerous physiological and pharmacological properties. Structurally, they have a common catechol ring (1, 2-dihydroxybenzene). Several studies have reported that natural catechols from plant extracts such as catechins and polyphenols have many pharmacological effects including anti-inflammatory, antioxidant and neuroprotective effects (Graefe et al., 1999; Hollman and Katan, 1999; Wiseman, 1999). Our present results indicated that the anti-inflammatory activities of catechols were related to their structure. For example, 3-methylcatechol and catechol have higher anti-inflammatory activity than 4-methylcatechol or 4-*tert*-butylcatechol in microglia.

NF-κB is a key transcription factor, which is activated by several cellular signal transduction pathways that are associated with the regulation of cell survival, expression of proinflammatory cytokines and enzymes such as iNOS, IL-1β and TNF-α (Baeuerle and Henkel, 1994; Baldwin, 2001). The molecular mechanisms of NF-κB activation have been well studied, and they involve a cascade activation of cytoplasmic proteins and the ultimate nuclear translocation of p65 subunit of NF-κB (Delhase et al., 2000; Karin and Ben-Neriah, 2000). In the current study, it was found that catechols attenuated IκB degradation as well as nuclear translocation of p65 in BV-2 microglia. These results indicated that catechols inhibited gene expression of iNOS and TNF-α in microglia, the mechanism of which at least in part might involve the inhibition of NF-κB activation. Additionally, it was found that catechols inhibited LPS-induced activation of p38 MAPK in BV-2 microglia. The p38 MAPK has been implicated in the signal transduction pathways responsible for the increased iNOS and TNF-α gene expression in glial cells or macrophages (Park et al., 2007). In this study, it was also found that the specific p38 MAPK inhibitor SB203580 markedly reduced NO production in the LPS-stimulated BV-2 microglia cells (Fig. 2A). Taken together, our results suggest that inhibition of NF-κB and p38 MAPK may be a molecular mechanism underlying the anti-inflammatory effect of catechol and its derivatives in microglia. The putative mechanisms involved in the regulation of inflammation and neuroprotection are illustrated schematically in Fig. 7.

Microglia have been related to disease progression and pathology in several neuroinflammatory diseases such as Alzheimer's diseases, Parkinson's diseases and HIV dementia (Block et al., 2007). Microglia activation has both beneficial and harmful effects on neuronal injury in neurodegenerative diseases. Overactivation of microglia contribute to neurodegenerative processes through the production of various neurotoxic factors including free radicals and proinflammatory cytokines (Klegeris et al., 2007). In fact, a number of anti-inflammatory agents, which inhibited microglial activation or production of

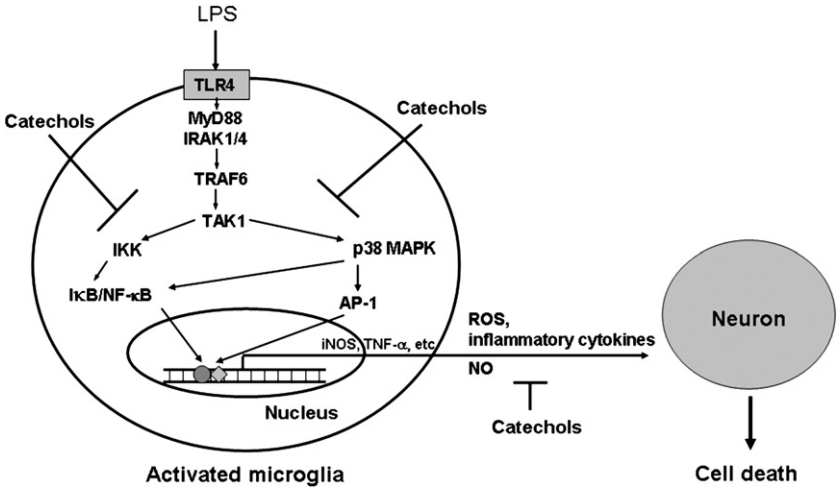

Fig. 7. Anti-inflammatory and neuroprotective mechanisms of catechols. Stimulation of TLR4 with LPS triggers the ubiquitination of TRAF6, which induces the activation of TAK1 through association of MyD88 and recruitment of IRAK1 and IRAK4. TAK1, in turn, phosphorylates both p38 MAPK and the IKK complex. The IKK complex then phosphorylates IκB, which leads to its ubiquitination and subsequent degradation. This allows NF-κB to translocate to the nucleus and induce the expression of its target genes such as iNOS and TNF-α, resulting in the production of NO, TNF-α and other proinflammatory molecules. These released proinflammatory mediators may lead to neuronal cell injury and death. Catechols may block NF-κB translocation and p38 MAPK phosphorylation to inhibit iNOS and TNF-α gene expression. In addition, catechols may directly scavenge the NO, thereby inhibiting microglial neurotoxicity. TLR4, Toll-like receptor 4; MyD88, myeloid differentiation primary-response protein 88; TRAF6, tumor necrosis-factor receptor-associated factor 6; IRAK1, IL-1R-associated kinase 1; TAK1, transforming-growth factor-β-activated kinase; IKK, inhibitor of nuclear factor-κB (IκB)-kinase.

proinflammatory mediators under the central nervous system disease conditions, attenuated neuronal degeneration (Esposito et al., 2007; Lee et al., 2003; McGeer and McGeer, 2007; Tikka et al., 2001). Thus, it is suggested that the search for the efficient anti-inflammatory compounds that attenuate microglial activation may lead to an effective therapeutic approach against many neurodegenerative conditions. The current study showed that catechols protected neuroblastoma cells against microglial neurotoxicity in a neuron/microglia co-culture (Fig. 6). The neuroprotective effects of catechol and its derivatives correlated well with their anti-inflammatory effects. Although the co-culture of the LPS-stimulated microglia with neuroblastoma cell line may not be the same as in the *in vivo* conditions, it partially reflects the pathological condition where activated microglia influences the death and survival of neuronal cells in neurodegenerative diseases. Further studies are, however, required to evaluate a neuroprotective property of the compounds in the animal models of neurodegenerative diseases, and to understand the precise molecular mechanisms of anti-inflammatory actions of the catechols *in vitro* as well as *in vivo*. The capacity of these compounds to cross the blood-brain barrier and achieve effective concentrations in the brain is also the subject of further studies. Moreover, it should be noted that catechols auto-oxidize at neutral pH, and this makes them highly unstable and likely unsuitable as potential drugs as they are. Nevertheless, this is the first study that has demonstrated anti-inflammatory effects of catechols in microglia, suggesting the neuroprotective effects of the catechol compounds against inflammation-mediated neurodegeneration. A similar anti-inflammatory effect of the compounds is expected in monocytes/macrophages in periphery. Future works along this line will give rise to a novel therapeutic use of the compounds and their stable derivatives for the treatment of neurodegenerative diseases and other inflammatory disorders.

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