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Neuroprotective effect of curcumin is mainly mediated by blockade of microglial cell activation

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Curcumin, the major yellow pigment in turmeric *(Curcuma longa)*, is a well-documented naturally-occurring anti-oxidant with numerous pharmacological activities such as anti-inflammatory, anti-carcinogenic and anti-bacterial effects. In this study, curcumin's neuroprotective effect was carefully examined using a coculture system, based on reports that curcumin-containing plants are neuroprotective. Coculturing neuronal cells and activated microglial cells enhanced dopamine-induced neuronal cell death from 30% up to 50%. However, curcumin did not protect dopamine-directed neuronal cell death and sodium nitroprosside (SNP)-induced NO generation, but only blocked activated microglial cell-mediated neuronal cell damage under inflammatory conditions. Indeed, curcumin blocked the production of pro-inflammatory and cytotoxic mediators such as NO, TNF-α, IL-1α, and IL-6 produced from Aβ(25-35)/IFN-γ- and LPS-stimulated microglia, in a dose-dependent manner. Therefore, our results suggest that curcumin-mediated neuroprotective effects may be mostly due to its anti-inflammatory effects.

1. Introduction

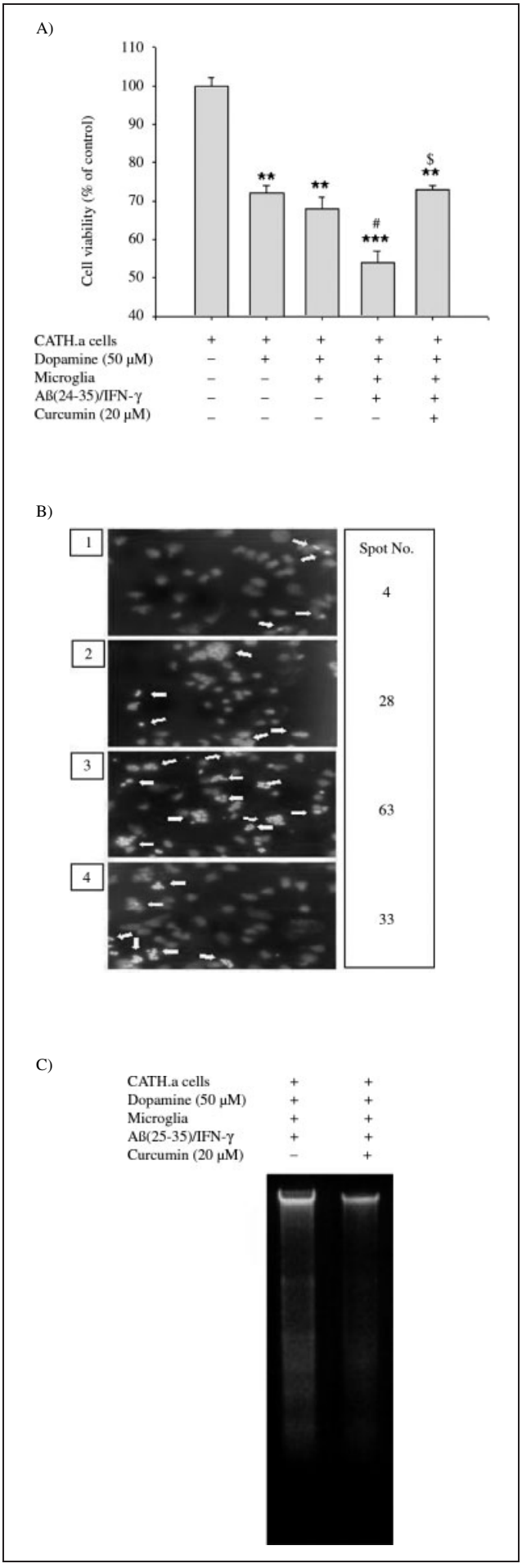
Neuronal cell degeneration in the brain leads to several serious diseases including Alzheimer's disease (AD) and Parkinson's disease (PD) (Sousa and Saraiva 2003). The neuronal degeneration is due to the neuronal cell death by various pathological reasons such as severe infection and the accumulation of environmentally toxic molecules (Pereira et al. 2004). The representative neuropathology-inducing molecules in AD and PD are amyloid β-peptide (Aβ), dopamine and some proinflammatory cytokines. The pathological molecules directly induce neuronal cell death via oxidative stress or indirectly stimulate glial cells such as microglial cells to produce neurotoxic inflammatory molecules such as reactive oxygen species (ROS), reactive nitrogen species (RNS), tumor necrosis factor (TNF)-α, and interleukin (IL)-1, thus leading to brain damage (Saito et al. 2005). Consequently, antioxidants (such as vitamin E, estrogens and melatonin) and anti-inflammatory drugs (such as aspirin) are known to be neuroprotective (Coskun et al. 2005).

Curcumin (diferulolmethane), the major yellow pigment in turmeric *(Curcuma longa)*, curry leaves *(Murraya koenigii)*, and mustard *(Brassica nigra)*, is a potent naturally-occurring antioxidant. Furthermore, it has been reported that curcumin exerts anti-inflammatory and anti-cancer effects (Sharma et al. 2005). The pharmacological merits of curcumin have led researchers to test its therapeutic efficacy against cancer, and inflammatory and neurodegenerative diseases. Indeed, the curative effects of the compound against the relevant diseases were revealed to be promising (Jones et al. 2000). Consequently, curcumin has been proposed to be therapeutically useful in various cancer and inflammatory diseases. In particular, it is noteworthy that this natural compound is applicable in various neurodegenerative diseases such as AD and PD. Therefore, to know the pathological mechanism of the diseases and to understand the action mode of action of curcumin regarding neurodegenerative diseases are important.

To evaluate the protective mechanism of a drug in neurodegeneration, a proper model which resembles real pathological situations is needed. In particular, it would be extremely useful to study neuronal cell damage *in vitro*, if normal brain-like conditions could be made available in a culturing system. For this purpose, we set up coculture conditions including neuronal cells (catecholamineergic or doparminergic neuronal cells) and glial cells (microglia) (Morita et al. 2003). Therefore, we have carefully evaluated curcumin's curative mechanism by which it protected neuronal damages shown in various animal models (Cole et al. 2005; Lim et al. 2001), since the exact molecular mechanism of curcumin's protective effects was not clear yet. Using the co-culture system, in fact, we have found that curcumin is able to protect from neuronal cell damage by anti-inflammatory activity, but not by anti-oxidative effect.

2. Investigations, results and discussion

To study the neuroprotective mechanism of curcumin, neuronal cells (CATH.a cells) were cultured with microglial



cells exposed to stimuli known to induce neuronal cell death. As stimuli for inducing neuronal cell death, LPS or dopamine and Aβ(25–35) were employed in this study. This was based on the reports that 1) dopamine can specifically induce apoptosis of CNS-derived neuronal cells such as catacholamine-containing cells (Suri et al. 1993) by generating toxic metabolites such as dopamine quinone or dopamine semiquinone as well as hydrogen peroxide (Choi et al. 2003; Izumi et al. 2005) and 2) like LPS, Aβ(25–35), an active peptide derived from inflammation-inducing amyoloid β polypeptide, is able to act as a microglial cell stimulator together with IFN-γ, to produce toxic inflammatory mediators such as NO and TNF-α. These phenomena are found in most neurodegenerative diseases such as AD and PD and therefore should be considered when drugs are evaluated. Based on previous findings (Morita et al. 2003), we designed a co-culture system composed of neuronal (CATH.a cells) and microglial cells cultured in Transwell cell culture inserts and employed two pathological stimuli such as dopamine and Aβ(25–35)/IFN-γ targeted to neuronal cells and microglial cells, respectively. This system renders us focusing the neuronal cell (CATH.a cells) damage (assessed by MTT assay) caused by dopamine and inflammatory mediators released from Aβ(25–35)/IFN-γ-activated microglical cells. Exposure to dopamine to CATH.a cells triggered apoptosis of the cells and co-culture of the cells with activated microglial cells enhanced the cell death (Fig. 1). Therefore, the neuroprotective effect of curcumin was investigated by co-culturing in the presence or absence of dopamine and Aβ(25–35)/IFN-γ.

As shown in Fig. 1, dopamine treatment (50 μM) induced neuronal cell toxic effect up to 30%. However, co-culturing with microglial cells stimulated by both Aβ(25–35) and IFN-γ enhanced the cell damage up to 55%. The neuronal cell death was induced by neuronal cell apoptosis. Both *in situ* DAPI staining (Fig. 1B) and *in vitro* DNA fragmentation (Fig. 1C) phenomena were clearly found in the apoptotic death of CATH.a cells. However, curcumin treatment significantly reduced neuronal cell death. Significantly, the protective level by curcumin exposure was not completely restored, even at non-toxic higher concentrations (up to 20 μM) (Fig. 1A), suggesting that there is a limited protection of neuronal cells by curcumin. To clarify the reason for the phenomenon, we next examined the

Fig. 1:
Effect of curcumin on neuronal death induced by activated microglia and dopamine. A) Coculture effect: Microglial cells pretreated with curcumin (10 μM) for 2 h were stimulated with 50 μg/ml of Aβ(25–35) plus 100 U/ml of IFN-γ. After 12 h, inserts were placed into the wells containing CATH.a cells (1×10^5 cells/well) pretreated with dopamine (50 μM) for 3 h. The viability of co-cultured cells was assayed by MTT assay after 24 h incubation. Results are the means ± S.E. of three independent experiments performed with triplicates. **: $p < 0.01$ and ***: $p < 0.001$ vs normal, #: $p < 0.05$ vs coculture group, and $: $p < 0.05$ vs control coculture groups. B) Immunostaining: Microglial cells pretreated with curcumin (10 μM) for 2 hr were stimulated under the same conditions. The co-cultured CATH.a cells were stained with Hoechst 33258 (bis-benzimide) for nuclear staining after 24 h. Potential apoptotic nuclei are indicated by arrows. 1. Normal CATH.a cells, 2. dopamine-treated CATH.a cells, 3. dopamine-treated CATH.a cells + activated microglial cells, 4. dopamine-treated CATH.a cells + activated microglial cells + curcumin (10 μM) C) DNA fragmentation: DNA was isolated from the co-cultured CATH.a cells (2×10^5 cells) damaged by dopamine and immunostimulated microglial cells, using phenol: chloroform : isoamylalcohol (25 : 24 : 1) mixture. The isolated DNA was electrophoresed in 1.5% agarose gel in TAE buffer. Data were a representative similarly obtained from 3 independent experiments

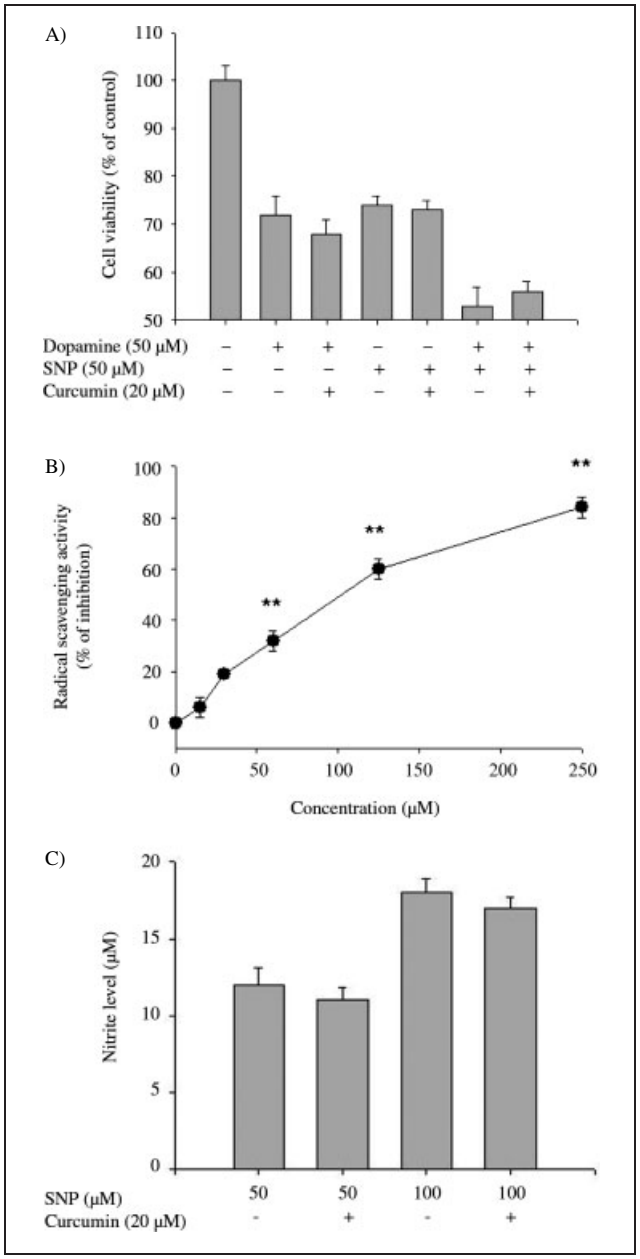


Fig. 2: Effect of curcumin on dopamine and/or SNP-induced CATH.a cell death, scavenging activity assessed by DPPH assay and SNP-released $NO^{\cdot}$. A) Cells were treated with dopamine (50 μM) and/or SNP (50 μM) and then after 24 h, cell viability was assayed by MTT assay described in Experimental. B) Antioxidant activity: Acetate buffer (10 mM, pH 5.5) and various concentration of curcumin were mixed and DPPH assay was carried out as described in Experimental. C) Cells were treated with SNP (50 or 100 μM) and then after 24 h, nitrite level in culture supernatant was assayed by Griess assay described in Material and Methods. Results are the means ± S.E. of three independent experiments performed with triplicates. **: $p < 0.01$ vs control group

potential mechanism of the curcumin-mediated partial protection against neuronal cell death. Because neuronal cell death was induced by two factors, dopamine and inflammatory toxic molecules such as NO, we first investigated if curcumin prevents dopamine-induced cytotoxicity. As shown in Fig. 2, dopamine treatment was cytotoxic up to 30% of cultured CATH.a cells. In particular, when dopamine-treated CATH.a cells were cultured in the presence of NO donor, SNP, the cytotoxicity of the cells increased by 50%. Although we cannot exclude other effects derived from NO itself such as alteration of intracellular signaling and gene expression pattern (Kim and Lee 2007; Song et al. 2007), the enhancement of neuronal death by a NO donor may be due either to up-regulating the formation of toxic dopamine oxidative products, as reported previously (Choi et al. 2003; Izumi et al., 2005) or independently activating the pro-apoptotic pathway by NO-derived toxic molecules such as peroxynitrite via releasing $NO^{\cdot}$ (Choi et al. 2002). Indeed, 50 and 100 μM of SNP released 12 and 18 μM of nitrites, respectively. However, curcumin never affected dopamine- or SNP-induced cytoxicity (Fig. 2), suggesting that curcumin is not able to neutralize the reactivity of the toxic molecules. Indeed, curcumin did not display a strong anti-oxidative activity with IC_{50} value of 91.3 μM, as assessed by DPPH assays (Fig. 2B). The SNP-released NO generation (assessed by nitrite determination) was not diminished by this compound as well (Fig. 2C). Therefore, these data suggest that curcumin's anti-oxidative property does not contribute to protection against neuronal cell death induced by reactive radicals.

Since curcumin blocked additional cell death mediated by co-culture of activated microglial cells, we next evaluated as to what curcumin conducted toward cytotoxic function by microglial cells in co-culture conditions. To do this, we first examined the cytotoxicity of curcumin itself under the conditions. Curcumin was non-cytotoxic up to 20 μM (Fig. 3). The pathological roles of microglial cells are especially evident in many neurodegenerative diseases (Minghetti 2005). In particular, the activated microglial cells were found at the damaged neuronal sites in AD and PD. The cells are known to be activated by bacterial endotoxin, some viruses, and neuronal cell-originated Aβ peptide, and eventually to be involved in inflammatory processes in damaged brain (Sargsyan et al. 2005). Furthermore, IFN-γ-producing T cells have also been detected in significant numbers in AD (Benveniste et al. 2004), and some damaged neurons are known to produce IFN-γ, as well (Vikman et al. 2001). Therefore, under such conditions, the activated microglial cells seem to be the major cells responsible for making neurodegenerative environments. Indeed, our co-culture condition included various pathological stimuli to activate the microglial cells and in turn the cells produced various pro-inflammatory molecules such as cytokines and NO up to 10- to 100-fold

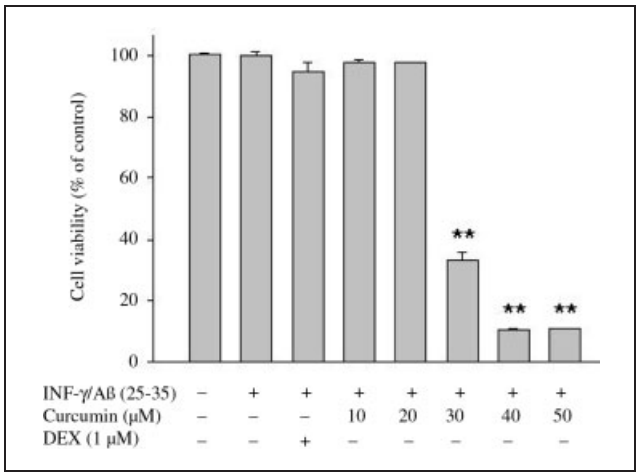


Fig. 3: Effect of curcumin on the viability of microglia stimulated with Aβ(25–35) and INF-γ. Cells pretreated with the indicated concentrations of curcumin were stimulated with Aβ(25–35) (50 μg/ml) plus IFN-γ (100 U/ml) for 24 h. Cell viability was measured by MTT assay as described in Experimental. Results are the means ± S.E. of three independent experiments performed with triplicates. **: $p < 0.01$ vs control group

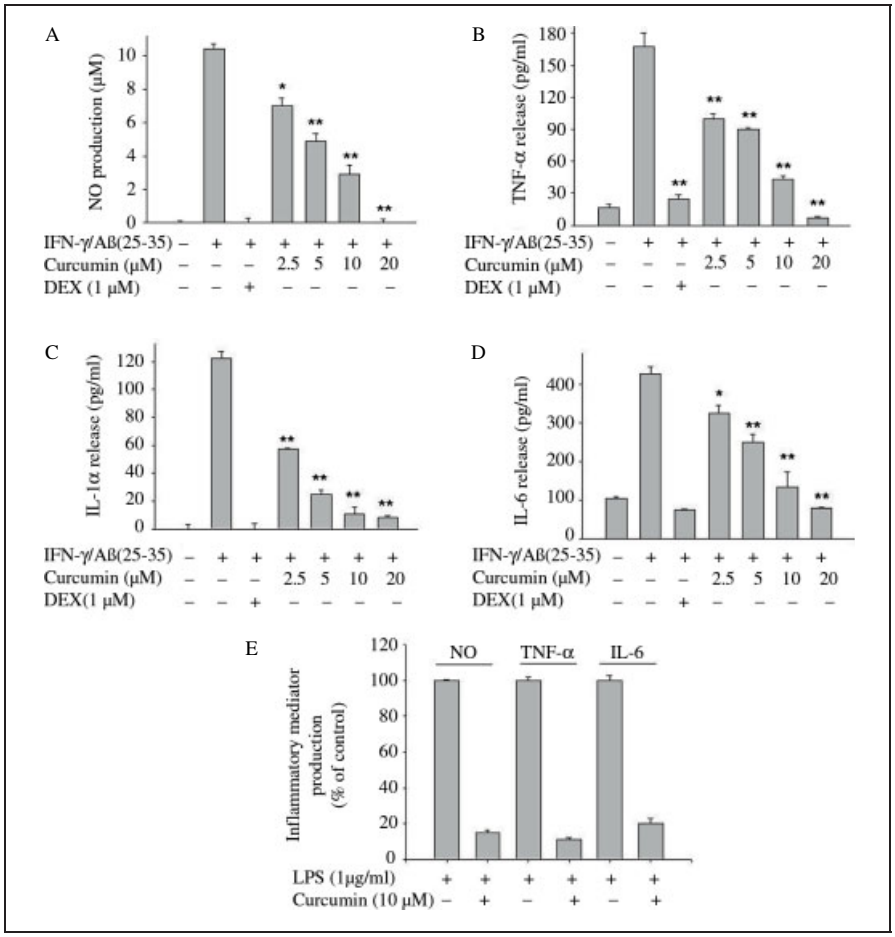


Fig. 4:
Effect of curcumin on inflammatory mediators (pro-inflammatory cytokines and NO) in Aβ(25–35)/IFN-γ-activated microglial cells. Cells pretreated with the indicated concentrations of curcumin or dexamethasone (DEX: 1 μM) were stimulated with Aβ(25–35) (50 μg/ml) plus IFN-γ (100 U/ml) or LPS (1 μg/ml). Cultured medium was collected after 24 h (A, E). Nitrite accumulation was determined by the Griess reaction as described in Experimental (B, C, D, E). The amount of TNF-α, IL-1α and IL-6 released was measured by ELISA. Results are the means ± S.E. of three independent experiments performed with triplicates. *: $p < 0.05$ and **: $p < 0.01$ vs control group

(Fig. 4). The conditions were decided by dose- and time-dependency test of Aβ(25–35) and IFN-γ in cultured microglial cells (data not shown). NO production was increased only when the IFN-γ was cotreated, while Aβ(25–35) alone did not stimulate the NO production (data not shown). The production of NO was dramatically increased when the concentration of Aβ(25–35) was elevated up to 50 μg/ml. Aβ(25–35) did not affect the cell viability regardless of the presence of IFN-γ (Fig. 3). Therefore, we investigated if curcumin modulates the functional activation of microglial cells, leading to neuronal cell-death.

Curcumin dose-dependently blocked the production of those molecules from activated microglial cells similar to dexamethasone (Fig. 4). Although curcumin appeared to more strongly block IL-1α production, the inhibition pattern of the products looked very similar. Thus far, it has been found that curcumin is able to block numerous pro-inflammatory and toxic molecules in macrophages and microglial cells via suppressing the activation of transcription factors (such as NF-κB, AP-1 and STAT) and their activation pathways linked to mitogen-activated protein kinases (such as ERK, p38, and JNK), and Janus kinases (Kim et al. 2003, 2005). Considering all this, the anti-inflammatory effect may be a critical property in curcumin's neuroprotection. That is, the scavenging effect (anti-oxidative activity) of curcumin against the reactivity of toxic radicals and the anti-apoptotic property against toxic radical-induced programmed cell death may not contribute to attenuate neuronal cell death; rather, it appears to interrupt the biosynthetic pathway of neuronal cell apoptosis-inducing molecules via its anti-inflammatory effect.

How can we then explain its protective effect in some neurodegenerative animal models induced by oxidative damage (Calabrese et al. 2007; Ghoneim et al. 2002; Zbarsky et al. 2005) should be clarified. One of putative mechanisms for solving this question seems to be its direct chelating activity of heavy metals (such as lead, copper and cadmium, etc.), involved in damaging the brain (Baum and Ng 2004; Daniel et al. 2004; Shukla et al. 2003). Indeed, numerous lines of evidence indicate that various metals are major pathological components leading to neuronal diseases. For examples they act as toxic catalysts in the oxidative deterioration of biological macromolecules and also induce Aβ aggregation in various neuronal disease models such as AD and PD, by directly or indirectly generating free radicals (Hermes-Lima et al. 1991; Stohs and Bagchi 1995). Under inflammation and acidosis of the AD brain, the release of copper and iron from melalloproteins is promoted. Interestingly, curcumin has been reported to suppress metal ion-induced lipid peroxidation and tissue damage in brain (Baum and Ng 2004; Daniel et al. 2004; Shukla et al. 2003). In particular, the curcumin's effects were also found to be due to chelating these toxic metals *in vivo* (Sumanont et al. 2006; Vajragupta et al. 2003). Considering the fascinating activities by curcumin, a chemical interaction between curcumin and metal ions may contribute to protect neuronal cells from oxidative damage.

In conclusion, co-culturing neuronal cells and activated microglial cells enhanced dopamine-induced neuronal cell death up to 50%. However, curcumin protected against neuronal cell damage mediated only by inflammatory processes. That is, curcumin blocked the production of pro-inflammatory mediators such as NO, TNF-α, IL-1α, and

IL-6 produced from LPS or Aβ(25–35) plus IFN-γ-stimulated microglia, in a dose-dependent manner.

Several ways to improve curcumin's poor bioavailability have been proposed in which some metabolizing enzyme inhibitors (eg. piperine) were co-administered to interrupt the rapid metabolism of curcumin in liver and intestinal wall (Shoba et al., 1998), so as that the therapeutic values of curcumin have been increased the. Curcumin's therapeutic potential can be enhanced if curcumin is co-administered with a drug which has strong anti-apoptotic and anti-oxidative effects. Thus, further studies will seek to find optimal partners for curcumin in the treatment of neurodegenerative disorders. Although it is known that curcumin blocks inflammatory responses in microglial cells, the fact (demonstrated by a co-culture system) that the anti-inflammatory effect is the main pharmacological mode of action of curcumin as a neuroprotextor could improve our knowledge in understanding curcumin's ethnopharmacology.

3. Experimental

3.1. Materials

Curcumin (purity: 98%, stock solution: 10 mM in EtOH) was isolated from turmeric *(Curcuma longa),* a common ingredient in Indian cookery and medical concoctions. Amyloid β-peptide 25–35 (Aβ25–35), curcumin (as a standard compound), dopamine, dexamethasone, lipopolysaccharide and sodium nitroprusside (SNP) were purchased from Sigma-Aldrich Chemical Co. (MO, USA). Minimum essential medium (MEM), neurobasal A medium, RPMI 1640 medium, Fetal bovine serum (FBS), Interferon-γ (recombinant rat IFN-γ, expressed in *E. coli*), Penicillin-streptomycin antibiotics, and Trypsin-EDTA were purchased from Life Technologies (NY, USA). IL-1, IL-6 and TNF-α ELISA kits, Western blotting detection system and ECL Plus™ were purchased from Amersham-Pharmacia Biotech. Ltd (Buckingamshire, UK). Protein assay kit was purchased from Bio-Rad Lab (CA, USA). Co-culture system, Transwell was purchased from Costa (USA). All other chemicals were obtained from Sigma-Aldrich Chemical Co. (MO, USA).

3.2. Primary microglial cell culture

Experimental protocols were approved by the Animal Care and Use Committees, National Institute of Toxicology Center, and conformed to regulations detailed in the National Institutes of Health publication Guide for the Care and Use of Laboratory Animals (revised in 1996). All efforts were made to minimize the suffering and the number of animals used according to the above-mentioned guidelines and derived guidelines on the ethical use of animals. Harlan Sprague-Dawley rats were purchased from the Dae-Han/Biolink Experimental Animal Center (Daejeon, Korea). Microglia were cultured from the cerebral cortices of 1 to 3 day old Harlan Sprague-Dawley rats by a modification of Park's method (Park et al. 1999).

3.3. CATH.a cell culture

A catecholaminergic cell line, CATH.a cells (Suri et al. 1993), was maintained by culturing in RPMI 1640 medium, supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) and 1% (v/v) streptomycin/penicillin, in a CO_2 incubator (5% CO_2-95% humidified air) at 37 °C.

3.4. Cell viability

Microglial cells were seeded on 24-well plates and cultured for 24 h. After treatment with Amyloid β peptides 25–35 (Aβ25–35) plus IFN-γ and curcumin (2.5–10 μM), cells were incubated for 24 h at 37 °C CATH.a cells were seeded on 12-well plates and cultured for 24 h and treated with various concentrations of dopamine and/or nitric oxide donor, Sodium nitroprusside (SNP). After 24 h, 1 ml of fresh MEM media and 100 μl (0.5 μg/ml) of 3-(4.5-dimethyl-thiazol-2-yl)-diphenyl tetrazolium bromide (MTT) were added to the plates, and incubated for at least 4 h at 37 °C. Following removal of media and MTT solution, 500 μl of dimethylsulfoxide (DMSO) was added to dissolve the remaining MTT-formazan crystal. The absorbance was measured at 570 nm.

3.5. Co-culture of microglia and CATH.a cell

Microglia cells on the microporous membrane of Transwell cell-culture inserts were cultured for 24 h. Cells pretreated with curcumin (2.5–10 μM), NMMA or dexamethasone for 2 h were stimulated with 50 μg of Aβ(25–35) plus 100 U of IFN-γ. After 24 h, the nitrite level was measured and then, inserts were placed into wells containing CATH.a cells (1×10^5 cells/well). Immunostimulants, Aβ(25–35) and IFN-γ, were not added to the co-culture media. CATH.a cells were pretreated with dopamine (50 μM) for 3 h before starting the co-culturing.

3.6. DAPI staining

To assess the nuclear morphology, co-cultured cells in a 12 well-plate were stained with bisbenzimide solution (Hoechst 33258; Sigma). Bisbenzimide (0.1 μg/ml) was dissolved in PBS/glycerol (1 : 1) solution. After rinsing with PBS, the cells were fixed by 4% paraformaldehyde in 0.1 M phosphate buffer and two drops of bisbenzimide solution were added. The cells were observed under a fluorescence microscope with an excitation wavelength of 365 nm.

3.7. DNA fragmentation

To approximately 2×10^5 cells 500 μl of lysis buffer (5 mM Tris, 20 mM EDTA and 0.5% Triton X-100, pH 8.0) were administered, scraped with cell scraper, and transferred to an Eppendorf-tube. The cells were pelleted by centrifugation at 12,000 × g for 5 min at 4 °C, and 5 μl of proteinase K was added as reported previously (Cho et al., 2004). The isolated DNA was examined by electrophoresis in 1.5% agarose gel in TAE buffer. The DNA in the gel was visualized under UV light after staining with 5 μg/ml of ethidium bromide (EtBr).

3.8. Determination of anti-oxidative effect

A DPPH decoloration assay was performed by a previously described method (Cho et al. 2006). The quenching of free radicals by each fraction and standard compound was evaluated spectrophotometrically at 517 nm against the absorbance of the DPPH radical. A freshly prepared DPPH solution (20 μg/ml) was used for the assays.

3.9. Measurement of nitrite

Microglia (4×10^5 cells/well) plated into 24-well plates were treated with curcumin (2.5–10 μM) and dexamethasone and after 2 h, stimulated with 50 μg of Aβ (25–35) and 100 U of IFN-γ or SNP (50 or 100 μM). After 24 h, the amount of nitrite converted from NO was determined by the Griess reaction (Cho et al. 2000).

3.10. Assay for proinflammatory cytokines

TNF-α, IL-1α and IL-6 contents were assayed using an ELISA kit. Briefly, microglia (4×10^5 cells/well) plated into 24-well plates were treated with curcumin (2.5 ~ 20 μM) and NOS inhibitor, dexamethasone, and after 2 h, stimulated with LPS (1 μg/ml), 50 μg of Aβ(25–35) and 100 U of IFN-γ. After 24 h, in microtitre plate coated with TNF-α, IL-1α, and IL-6 antibody, the amount of the cytokines was determined in 50 μl of the culture supernatants.

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