Neuroprotective activities of *Citrus sunki* peel extract against neuroinflammation and microglia-mediated neurotoxicity

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Abstract

It has been reported that neuroinflammation is associated with the pathophysiology of many neurodegenerative disease, including Alzheimer's disease (AD), and the treatments of anti-inflammatory drugs show the progress of these disease. In this study, we investigated the anti-inflammatory and neuroprotective effects of ethanol extracts of *Citrus sunki* peel(CPE) in BV2 microglia and HT22 neurons. It was found that CPE significantly suppressed LPS-induced NO production, pro-inflammatory cytokine IL-6 and proinflammatory enzyme cyclooxygenase 2 (COX-2) in BV2 microglia. It also reduced LPS-induced NF-kB translocation and phosphorylation of MAP kinase (ERK, JNK, and p38). On the other hand, CPE significantly attenuated the neurotoxicity induced by BV2 microglia. Taken together, these results suggest that CPE has therapeutic potentials against neurodegenerative diseases via regulation of microglial activation. (J Med Life Sci 2012;9:49-53)

Key Words : Citrus, neuroinflammation, neuroprotection

Introduction

Citrus fruit peel has been used in traditional Asian medicine for centuries and flavonoids citrus fruit peels have significant anti-oxidation properties[1]. However, neuroprotective activities of Citrus fruit peel has not been reported.

It has been studied that neuroinflammation play a key role in many neurodegenerative diseases. Microglia are believed to play an important role in the pathway that leads to inflammation-mediated neuronal cell death in a number of neurodegenerative diseases[2]. Activated microglia release neurotoxic and proinflammatory factors, including nitric oxide (NO), prostaglandinE2 (PGE2) and proinflammatory cytocytokines including IL-6, IL-1β, TNF-α. Uncontrolled activation of microglia may cause neuronal damage through the overproduction of proinflammatory substances, including proinflammatory cytokines, complement proteins and reactive proteinases, in neurodegenerative diseases[3]. Therefore, controlling microglial activation may have potential therapeutic options for the treatment of various neurodegenerative conditions[4,5].

In this study, we examined the effects of CPE on microglia-induced neurotoxicity, microglia activation. The

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results suggest CPE not only effectively suppressed LPS-induced microglia activation but also effectively protected neuron cells against microglia-induced neurotoxicity. In summary, CPE has neuroprotective acitivity and therapeutic potentials against neuroinflammation and neurodegenerative diseases via regulation of microglial activation.

Materials and methods

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Cell culture

HT22 cell (neuron) and BV2 cell (microglia) were maintained at 37℃ in the incubator with a humidified atmosphere of 5% CO2, and cultured in Dulecco's modified Eagle medium supplemented with 10% (v/v) fetal bovine serum and 1% penicillin streptomycin .

Reagents

CPE was prepared from the peel of Citrus sunki Hort. ex Tanaka, as described previously[6]. Briefly, the peels from mature fruits of C.sunki were obtained from Seogwipo-si on Jeju island, South Korea in September, 2008. DMEM, FBS, penicillin, and streptomycin were obtained from Invitrogen

(Carlsbad, CA, USA). All other reagents were purchased from Sigma-Aldrich (St Louis, MO, USA), unless indicated.

MTT assay

The effect of CPE on the viability of the cell was determined using the [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium] bromide (MTT) assay. Cells were seeded on 24-well plates at 1×105 cells per well containing 500 ml medium. After treatment, 200 ml MTT (2 mg/ml) was added to each well and the mixture further incubated for 2 hrs. The liquid in each well was then aspirated and 500 ml dimethyl sulfoxide (DMSO) was added, mixed thoroughly on a shaker and absorbance was subsequently read at 540 nm using a microplate reader (Model 550; Bio-Rad, USA).

Measurement of NO production

BV-2 microglial cells were seeded on 24-well plates at 1×105 cells per well containing 500 ml medium, then incubated with LPS (200 ng/ml) were incubated for 24 h in the presence or absence of CPE. Nitrite in culture supernatants were measured by adding qual amounts of Griess reagent (1% sulfanilamide and 0.1% N-[1-naphthyl]-ethylenediamine dihydrochloride in 2.5% phosphoric acid). Samples were incubated at room temperature for 10 minutes and absorbance was subsequently read at 540 nm using a microplate reader.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from cells by the TRIZOL Reagent (invitrogen, Molecular Research Center) method following the manufacturer's instructions. The RNA extraction was carried out in an RNase-free environment. Briefly, 1 mg of total RNA was incubated in a tube containing 100 U of SuperScript II Reverse Transcriptase (Invitrogen), 100 U RNaseOUT (Invitrogen), 250 mM dNTPs, and 0.5 mM oligo(dT)12-18primerfor15minutesat70℃. The reaction was terminated by heating the mixture at 70℃ for 5 minutes followed by cooling on ice. The RNA strands in the RNA-DNA hybrid were removed by adding 10 U RNaseH (Invitrogen) and incubating for 45 minutes at 42℃. Polymerase chain reaction (PCR) was performed in a total volume of 25 ml containing 3 ml cDNA, 200 mM dNTPs, 20 pmol of each primer pair, and 3 U Taq DNA polymerase (iNtRON, USA) in Taq DNA polymerase reaction buffer as provided by the supplier. A total of 30 cycles were run

under the following schemes; denaturing at 94℃ for 40 seconds, primer annealing at 50℃ (TNF-a) and 55℃ glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and an extension step at 72℃ for 1 minute in the Peltier Thermal Cycler (PTC)-100 (BIO-RAD). The following sets of primers were used in the PCR amplifications: GAPDH (forward 5' -GTCCACATTGTTGCCATCAACGAC-3', reverse 5' -TTTCTCGTGGTTCACACCCATCAC-3'), IL-6. PCR products were separated electrophoretically on 1% agarose gel, stained with ethidium bromide, and subsequently visualized under UV illumination. Optical densities of the PCR bands for IL-6 were quantified with an Image J analyzer (http://rsb.info.nih.gov/ij/) and normalized with those of GAPDH.

Western blot analysis

The cells were harvested, and washed twice with PBS. The harvested cells were then lysed on ice with RIPA buffer (10mM Tris-HCl; pH 7.4, 150mM NaCl, 1mM EGTA, 0.1% SDS, 1mM NaF, 1mM Na_3VO_4,1mMPMSF,1mg/ml aprotinin, and 1 mg/ml leupeptin) and centrifuged at 16000 g for 15 min. Supernatants were collected from the lysates and protein concentrations were determined. Aliquots of the lysates (20 μg of protein) were boiled for 15 min and electrophoresed in 10% sodium dodecysulfate-polyacrylamide gel. Blots in the gels were transferred onto nitrocellulose membranes, which were then incubated with primary antibodies. The membranes were further incubated with horseradish peroxidase (HRP)-conjugated anti-mouse IgG antibody or anti-rabbit IgG secondary antibody (Vector Laboratories, USA) HRP-conjugated secondary antibodies. After several washes, the blots were developed using an enhanced chemiluminescence reagent (ECL, Amersham Biosciences, USA) according to the manufacturer's instructions. The quantification of relative band intensities from the results was determined by Image J program.

Conditioned media treatment

For conditioned media experiments, HT22 neurons were plated at 24 well plates and after allowed to settle for 24 h before replacement with conditioned media. BV2 microglia were treated with CPE for 1 h prior to 400 ng/ml LPS stimulation, respectively with LPS alone (LPS-CM) and incubation for 24 h. Then the BV2 microglia-conditioned media were collected and were transferred respectively to HT22 neurons for a further 24 h. The survival of HT22

neurons were determined by MTT assay.

Statistical analysis

All the values were represented as means ± standard error of mean (SEM). The results were subjected to an analysis of the variance (ANOVA) using the Student's- test to analyze the differences. P<0.05 were considered to be significant.

Results

Effects of CPE on the survival of BV2 microglia and HT22 neurons.

First, the in vitro cytotoxicity of CPE was tested in BV2 and HT22 cells. The cell viability was tested at various concentrations of CPE in BV-2 and HT22 cells by MTT assay (Fig. 1). Results showed that CPE alone at concentrations 50, 100, 200 and 400 μg/ml had no significant effect on the viability of cells. At concentrations 1 mg/ml, CPE significantly reduced the cell viability of BV2 and HT22 cells.
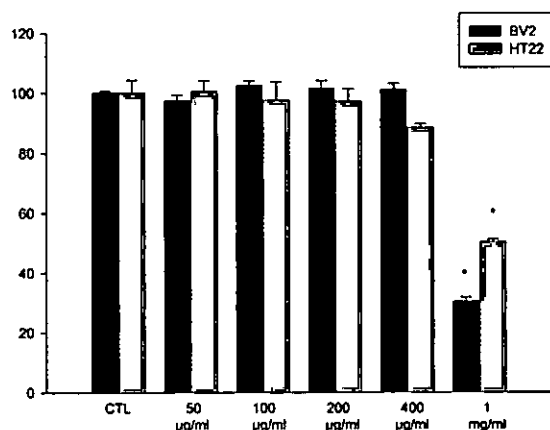

Figure 1. Cytotoxicity test of the CPE in BV2 microglia and HT22 neurons. Cells were treated with citrus peel extract for 24 h at the indicated concentrations. The cell viability was determined by MTT assay. The results are mean±SEM(n=3). *P<0.05.

Inhibitory effect of the CPE on LPS-induced neuroinflammation in BV2 microglia.

NO is produced endogenously by iNOS with a wide range of physiological and pathophysiological action. Increased NO and iNOS production has been most widely used

representative indicator of microglial activation among many inflammatory mediators. After pretreatment with CPE for 1 h, BV2 microglia were treated with LPS (200ng/ml) in the presence or absence of CPE for 24 h. CPE treatment significantly reduced LPS-induced elevation of nitrite concentration in the medium in dose-dependent manner by 28.22 ± 14.48%, 31.38 ± 18.73%, 39.36 ± 13.43% (Fig. 2A), compared to the LPS treated group. Therefore, in this study, we used CPE at concentrations 200 μg/ml, which no effect on the cell viability for further studies. As shown by western blot analysis, CPE was also suppressed iNOS expression (Fig. 2B). It is also effective in the inhibition of proinflammatory cytokine production through the regulation of the gene transcriptional levels of IL-6 in activated microglia (Fig. 2C). COX-2 is the predominant cyclooxygenase at sites of inflammation, we studied the ability of CPE to influence the LPS-induced production of COX-2. LPS treatment significantly increased the expression of COX-2 and this expression was markedly attenuated in BV2 microglia pretreated with CPE (Fig. 2D). These results suggest that CPE attenuated IL-6 production through the regulation of COX-2 protein levels in activated microglia.
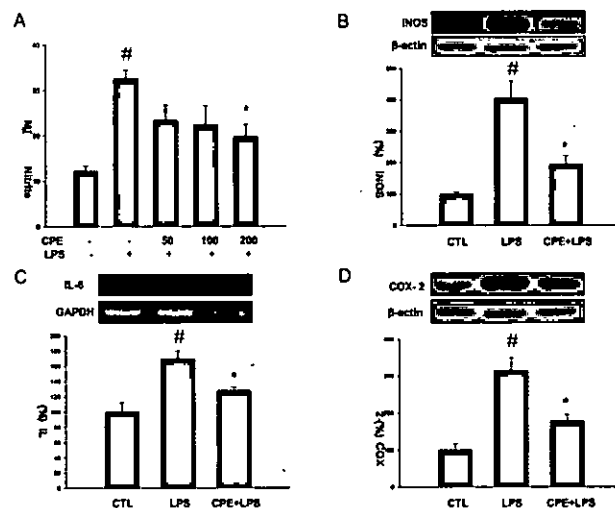

Figure 2. Inhibitory effect of CPE on LPS- stimulated NO release and iNOS expression in BV2 microglia. BV2 cells were treated with CPE at indicated concentration for 1 h and stimulated with LPS (200 ng/ml) for 24 h. Culture supernatants were collected in order to measure NO concentrations using the Griess reaction (A) and Cell lysate were extracted, and protein levels of iNOS protein was determined by Western blotting (B). IL-6 mRNA expression was determined by RT-PCR (C) and COX-2 protein was analyzed by Western blotting (D). The data were replicated in three repeated independent experiments. #, p<0.005 as compared to unstimulated control group and *, p<0.05 as compared to LPS alone-treated group.

Inhibitory effect of CPE on MAPK phosphorylation and IκB degradation.

MAPKs pathway was involved in the regulation of microglial inflammation. Following a 24 h CPE pretreatment, BV-2 microglial cells were treated with LPS (200 ng/ml) in the presence or absence of CPE (200 μg/ml) for 30 min. After LPS stimulation, phosphorylations of mitogen-activated kinases (MAPKs) including ERK, p38 and JNK were investigated to determine whether they were involved in the action mechanism of CPE. As a result, CPE remarkably attenuated LPS-induced phosphorylation of ERK1/2, SAPK/JNK, and p38 MAPK by 22.43 ± 0.49, 31.24 ± 3.35 and 42.73 ± 9.07, compared to the LPS treated group (Fig. 3A-C), while their non-phosphorylated forms remained the same. This result indicates that signal transduction by three MAPKs might be effectively blocked by CPE in activated microglia.

NF-kB is a key molecule in microglia activation pathway, which is implicated in the transcriptional regulation of inflammatory mediators in LPS-stimulated BV2 cells. CPE inhibited LPS-induced degradation of IκB in cytosol (Fig. 3D). These results indicated the potential role of NF-kB in the possible mechanism of CPE in suppressing NO and proinflammatory cytokines in activated microglia.
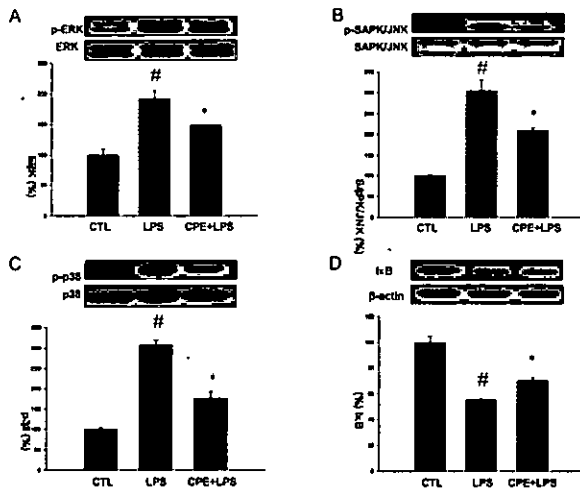
Figure 3. Inhibitory effect of CPE on the protein level of Iκ B in LPS-stimulated BV2 microglia. BV2 cells were treated with LPS for 30min in the presence or absence of CPE (200μ g/ml). CPE was pretreated for 24 h before LPS stimulation. The cellular proteins from the cells were used for the detection of Iκ B ERK(A) , SAPK/JNK (B), p38 MAPKs (C) and Iκ B(D) by Western blotting. #, $p<0.05$ as compared to unstimulated control group and *, $p<0.05$ as compared to LPS alone-treated group.

Neuroprotective effect of CPE on microglia-induced neurotoxicity in HT22 cells.

Because microglia activation and propagating neuron death are implicated in development of many diseases. next we examined the microglia-induced neurotoxicity in HT22 cells. First, BV2 micrglia were activated by LPS (400 ng/ml) for 24 h. Next, the cultured medium was used to culture HT22 neurons for another 24 h. The BV2 cultured medium was contains much neurotoxicity cytokines and NO and can induce neuronal cell death. CPE was also significantly increased microglia-induced neurotoxicity in HT22 cells (Fig. 4).
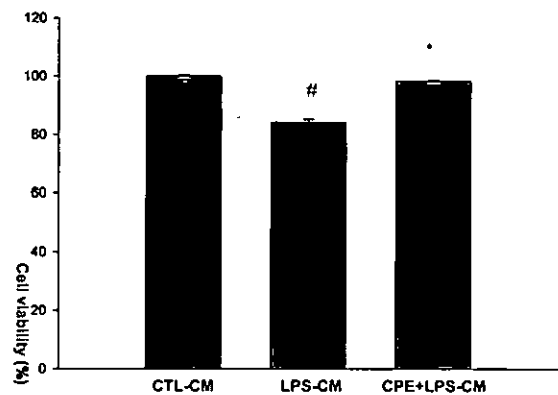
Figure 4. Neuroprotetive effect of CPE on cell survivalof neuronal cells treated with conditioned media from LPS-stimulated microglia. BV2 cells were treated with CPE at indicated concentration for 1h and stimulated with LPS (400 ng/ml) for 24 h. The conditioned media was then transferred to HT22 neurons with equal volume of fresh media for a further 24 h, respectively. HT22 cell viability was assessed by MTT assay. #, $p<0.05$ as compared to unstimulated conditioned BV2 media group(CTL-CM)and *, $p<0.05$ as compared to LPS-stimulated conditioned BV2 media group(LPS-CM).

Discussion

Citrus has been used in traditional Asian medicine for centuries. Recently, the anti-inflammatory activities among 20 Citrus are significantly correlated with the content of nobiletin which was known as action compound of Citrus[6]. Nobiletin is a citrus polymethoxylated falvonoid extracted from Citrus and has several reported biological effect including cognition and memory[7-9].

A major focus on research neurodegenerative disease is to investigate the inflammatory processes. Inflammatory processes are associated with the pathophysiology of many

neurodegenerative diseases, including Alzheimer's disease (AD). It has been studied that abnormalities in the production of inflammatory mediators, such as NO and IL-6, may play roles in many inflammatory lesions. These inflammatory mediators are released in the brain following many different neuropathological conditions. Activated microglia contribute secondarily to inflammation-mediated tissue destruction via the released of cytokines such as IL-6, TNF-α. Among these mediators, NO and IL-6 have relations with iNOS and COX-2. COX-2 is the predominant cyclooxygenase at sites of inflammation[10] and was produced in macrophages and endothelial cells in response to various stimuli, such as neuronal activity, proinflammatory cytokines. In present study we examined the biological effects of CPE on the production of inflammatory mediators in murine macrophage BV2 microglia which was stimulated with LPS. We found that CPE significantly inhibited LPS induced increase of NO and IL-6. And it also blocked LPS induced overexpression of iNOS, and COX-2. It was evidenced that MAP kinases play a key role in the regulation of cell growth and differentiation and in the control of cellular responses to cytokines and stresses, as well as in the activation of NF-κB[5]. To further understand the molecular mechanism of CPE activity in microglia, we examined the effect of CPE on the LPS-induced phosphorylation of MAP kinase and IκB in BV2 microglia using Western blot analysis. All of our results indicated that CPE effectively inhibited LPS-induced activation of BV2 microglia. The current study revealed the conditioned media from LPS-stimulated microglial cells was toxic to neuronal cells, which was similar to the recent studies[11].

In summary, the present study demonstrated that CPE has neuroprotective acitivity and therapeutic potentials against neuroinflammation via regulation of microglial activation.

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