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Original Research Article

Anti-inflammatory mechanism of *Isodon japonicas* (Burm) Hara on lipopolysaccharide-induced neuroinflammation

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Abstract

Purpose: *To investigate the anti-neuroinflammatory effects of Isodon japonicus (Burm.) Hara extract (IJE) on BV2 microglial cells. .*
Methods: *Cell viability was evaluated by MTT method. BV2 microglial cells were stimulated with lipopolyscarride (LPS, 1 μg/ml) and the effect of IJE on nitric oxide (NO) levels were measured using Griess assay. Immunoblot analysis was used to assess the effect of IJE on protein expression of inducible NO synthase (iNOS) expression. Tumor necrosis factor-alpha (TNF-α) cytokine production was evaluated by enzyme-linked immunosorbent assay (ELISA).*
Results: *Pretreatment of 100 mg/ml of IJE ($p < 0.001$) was inhibited nitric oxide (NO) by 1 ug/ml LPS-treated BV-2 cells. iNOS and TNF-α expression were attenuated by IJE concentration-dependently ($p < 0.001$ at 100 mg/ml). IJE scavenged 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radicals in a dose-dependently with half-maximal inhibitory concentration (IC_{50}) value of 46.5 μg/ml.*
Conclusion: *Data from this study indicate that IJE attenuates neuroinflammatory responses. The strong anti-oxidant effect of IJE modulates expression of inflammatory molecules at the transcription level, and TNF-α at post-transcription level.*

Keywords: *Isodon japonicas, Anti-oxidant, Neuroinflammation, BV-2 microglia, Nitric oxide*

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INTRODUCTION

Inflammation is often considered a universally harmful event. The presence of cytotoxic cytokines, invading immune cells, and tissue destruction reinforces the idea that inflammation is synonymous with tissue pathology. Neuroinflammation has been considered a driver of pathology and cognitive dysfunction for many years; however, not all inflammation results in the same outcome. The brain is sensitive to a wide variety of inflammatory stimuli that can result in different outcomes depending on the type of exposure, environment, and underlying pathological processes. Neuroinflammation is a result of an innate immune response in the CNS. Being a “semi-immune privileged organ” the brain and spinal cord have many resident cell types capable of producing immune related factors that can trigger neuroinflammation (1). Astrocytes, microglia, and even neurons are all crucial players in maintaining tissue homeostasis, and in times of pathology, each of these cell types can contribute to an

inflammatory response through cytokine secretion. Of these cells, microglia are thought to be the principle cell type that is pivotal in immune surveillance and maintaining homeostasis in the CNS. Such control is vital in the brain, which is the master regulator of many bodily functions. In particular, neurons have been shown to be extremely sensitive to neuroinflammation and can alter their connectivity depending on their environment (2). Microglia, the immune cells in the central nervous system (CNS), when activated produce several inflammatory mediators [3,4], and may lead to progressive damage process in a number of neurodegenerative disorders including [4]. It is well known that microglia can be activated by lipopolysaccharide (LPS) and is recognized as a beneficial tool for studying neuroinflammatory mechanisms [5,6]. Thus, development of new anti-neuroinflammatory agents that reduce microglial activation and their proinflammatory responses is considered an important therapeutic strategy for neuroinflammatory disorders. *Isodon japonicus*, a perennial plant grown extensively in Korean peninsula has long been used as a traditional medicine [7,8]. However, till date the effect of *Isodon japonicus* (IJE) on neuroinflammation and LPS-activated microglial neurotoxicity has not been documented. The aim of this study was to investigate whether *Isodon japonicus* exhibits protective effects on LPS-activated neuroinflammatory processes in BV-2 microglial cells.

EXPERIMENTAL

Plant materials and extraction

Isodon japonicus was purchased from a local market in Chungnam, Korea in August 2013 and was authenticated by Dr JB Kim, a taxonomist at Konkuk University, Glocal Campus, Republic of Korea, and a voucher specimen (no. SG-DKU2016) were stored in the herbarium of Dankook University, Korea for future reference. To obtain the *Isodon japonicas,* the material was washed in running tap water and chopped into smaller pieces. *Isodon japonicas* (200 g) was extracted with 1L of water at 70 °C for 2 h. Water extracts were filtered and evaporated to dryness. The dried material was re-dissolved in 0. 5 L of distilled water.

Determination of free radical scavenging activity

The anti-oxidant activity of the IJE was evaluated using the DPPH (Sigma-Aldrich, St. Louis, MO, USA). The radical scavenging capacity was determined by a reaction mixture constituted by aliquots of the IJE and a DPPH methanolic solution as described previously [14]. Furthermore, a sample solution of 30 µl, was added to 30 µL of DPPH (30 µM) in methanol solvent. After mixing vigorously for 15 s, the mixture was transferred into a 50 µL capillary tube and the free radical activity of each sample on DPPH was measured by JES-FA ESR spectrometer (Jeol Ltd, Tokyo, Japan).

Cell culture and viability assay

Murine BV-2 microglia cells were cultured in 5 % CO_2 at 37 °C supplemented with 5 % FBS + DMEM (Hyclone, Logan, UT, USA). In all experiments, cells were pre-treated with IJE at concentrations from 10 µg/ml to 100 µg/ml) for 1 h. And then the addition of 1 µg/ml of LPS in serum-free DMEM.

For viability assay, BV-2 cells were plated onto 96 well plates and exposed to IJE. MTT reagent was added to each 96 well plate then incubated for 2 h in at 37 °C. The supernatant was aspirated from the 96 wells and the blue formazan product obtained using DMSO. The samples in each of the 96 well were analyzed at 570 nm using Tecan microplate reader. Each experiment was conducted in triplicate. Percentage of the cell viability was convert the value to %, (OD sample-OD medium)/(OD control -OD medium) x 100 %.

Immunoblot analysis

BV-2 Cells were washed in cold PBS (PH 7.4) three times and were lysed in a cell lysis buffer containing 25 mM Tris-HCl, pH 7.4, 0.5 % (v/v) NP-40, 0.20 % sodium deoxycholate, 100 mM Sodium Chloride , 0.5 mM EDTA, 20 mM NaF, 1 mM Na_3VO_4 and protease inhibitor Complete Mini^{TM} cocktail (Roche, Mannheim, Germany) at 4°C. The lysate was purified by centrifugation at 10,000 *g* for 10 min at 4 °C.

Cell lysates were normalized for protein content using Pierce BCA reagent (Rockford, IL, USA). Equal amounts of each protein were loaded onto 10 % PAGE gels and were separated by standard SDS-PAGE procedure. And then, Proteins were transferred to a Nitro-cellulose membrane (Bio-Rad, Herculus, CA, USA) and blocked with 10 % non-fat dry milk in PBS (pH 7.4). To detect iNOS protein expression, the blots were probed with the anti- iNOS followed by the rabbit-secondary antibodies coupled to horseradish peroxidase (Bio-Rad, Herculus, CA, USA). The detection of β-actin antibody was used for an internal control. The complexed proteins on the membrane were detected by

chemiluminescence using the Pierce substrate on X-ray film. Anti-iNOS and anti-β-actin antibodies were purchased from Cell Signaling Technology Beverly, MA, USA).

NO assay

Production of nitric oxide was measured the levels of nitrite in the culture supernatant using colorimetric assay using Griess reagent [10]. Briefly, 2 x 10^5 cells/ml of BV-2 cells were seeded in 6-well plates in 200 µl complete culture medium and treated with the IJE at indicated concentrations for 30 min prior to stimulation with 1 µg/ml of LPS for 1 h. Culture supernatant (30 µl) was reacted with an equal volume of 0.1 % naphthylethylenediamine and 1% sulfanilamide in 5 % H_3PO_4 (Griess reagent) in 96-well plates at room temperature. Each samples of nitrite oxide concentrations were measured by using a standard solutions of sodium nitrite. The absorbance was determined at 540 nm in Tecan microplate reader.

TNF-α assay

Murine BV-2 microglia cells were cultured on 96 well plates (1 x 10^5 cells/well) and treated with the IJE at indicated concentrations for 1 h and stimulated with LPS (1 µg/mL). At 4 h treatment with LPS, the cells were harvested and the supernatants (SNT) were measured for TNF-α levels using a TNF-α mouse ELISA kits from Invitrogen (Carlsbad, CA).

Statistical analysis

All data are presented as mean ± SEM. Statistical significance (p < 0.05 for all analyses) was assessed by ANOVA using Instat 3.05 (GraphPad, San Diego, CA), followed by Student–Newman–Keuls analysis.

RESULTS

Effect of IJE on DPPH radical scavenging activity

As shown in Figure 1 A, IJE exhibited significant DPPH radical scavenging activity in a concentration-dependent manner showing a maximum effect at 200 µg/ml (p < 0.001). The half maximal inhibitory concentration (IC_{50}) of DPPH free radicals was approximately 46.5 µg/mll.

Effect of IJE on BV-2 cell viability

As shown in Figure 2, treatment with IJE at 10 - 200 µg/ml concentrations did not affect the cell viability nor ensured it exhibit any cytotoxicity on BV-2 microglia cells. The data indicated that the concentrations used in the study were safe to BV-2 microglial cells.

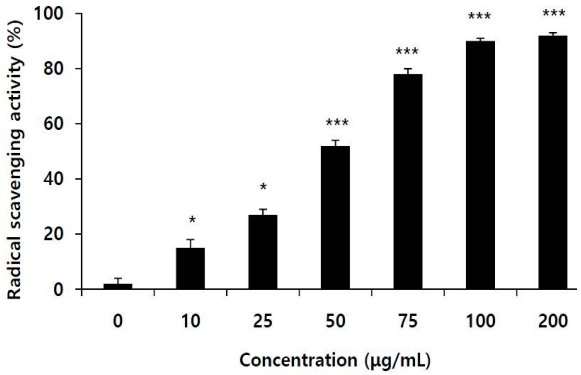

Figure 1: Effect of IJE on DPPH radical scavenging activity. The capacities to scavenge DPPH radicals by different concentrations of IJE. BV-2 cells were treated with or without IJE at the 10, 25, 50, 75, 100 and 200 µg/ml concentrations (). All data are presented mean ± S.E.M. (n = 4); *p < 0.05 and ***p < 0.001, compared with control group by Student t-test

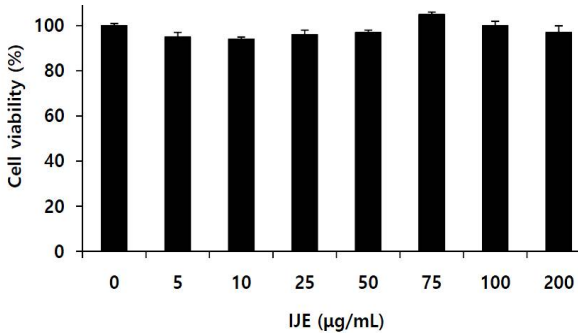

Figure 2: Effects of IJE on the viability of BV-2 microglial cells. Viability in IJE-treated BV-2 cells was evaluated using MTT assay. This results are depicted as percentage of control samples. All data are presented mean ± SEM (n = 4). IJE*: Isodon japonicas* extract

IJE attenuates NO production in activated BV-2 cells

As shown in Figure 3, BV-2 cells treated with 1 µg/ ml of LPS alone significantly increased in NO (nitric oxide) levels (p < 0.001). Pre-treatment with IJE at indicated concentrations significantly and dose-dependently suppressed the excessive release of NO in BV-2 cells (Figure 3). Significant effect was observed at a concentration of 100 µg/ml (p < 0.001

IJE attenuates iNOS expression in activated BV-2 cells

As shown in Figure 4, immunoblot analysis revealed that activation of BV2 microglia by lipopolysaccharide increased the protein

expression levels of iNOS. However, the increased expression of iNOS in activated BV-2 cells was suppressed at a concentration of 20 µg/ml and 40 µg/ml of IJE.

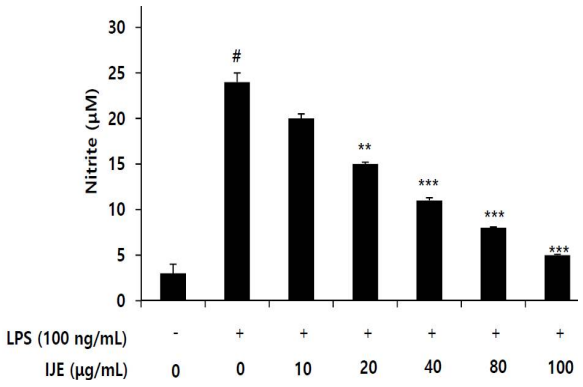

Figure 3: Effect of IJE on NO production in activated BV-2 cells. The nitrite in the culture supernatant was tested using Griess reagent. All data are presented mean ± SEM (n = 4); $^{\#}p < 0.001$, when compared with control; $^{**}p < 0.01$ and $^{***}p < 0.001$, when compared with LPS alone by Student t-test. (IJE*: Isodon japonicas* extract LPS: lipopolysaccharide).

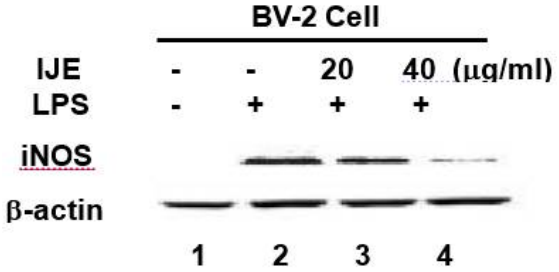

Figure 4: Effect of IJE on iNOS expressional levels in activating BV-2 microglial cells. The expression levels of iNOS protein in the LPS (1 µg/ml) - activated BV-2 cells by 40 and 80 µg/ml concentration of the IJE was measured by immunoblot analyses against anti-iNOS antibody. The internal control used was β-actin. (IJE: *Isodon japonicas extract;* LPS: Lipopolysaccharide; iNOS: Inducible nitric oxide).

Effect of IJE on TNF-α in activated BV-2 cells

Proinflammatory cytokine, TNF-α levels increased significantly after 1 µg/ml of LPS treatment when compared to those in LPS-untreated cells ($p < 0.001$) (Figure 5). However, IJE significantly attenuated TNF-α production in a dose dependent manner in activated BV-2 cells ($p < 0.01$ at 50 µg/ml and $p < 0.001$ at 100 and 200 µg/ml, respectively).

DISCUSSION

This is the first report to demonstrate that IJE markedly inhibited LPS-induced inflammatory responses in the BV-2 microglial cell line. NO production and iNOS expression in BV-2 cells were significantly inhibited by IJE in a concentration-dependent manner. This protective effect of IJE was also evidenced by inhibiting TNF-α release. Moreover, the cell viability assay showed that treatment with IJ alone at various concentrations (20 - 200 µg/ml) did not have cytotoxic effects, whereas IJE significantly inhibited the inflammatory mediators stimulated by LPS.

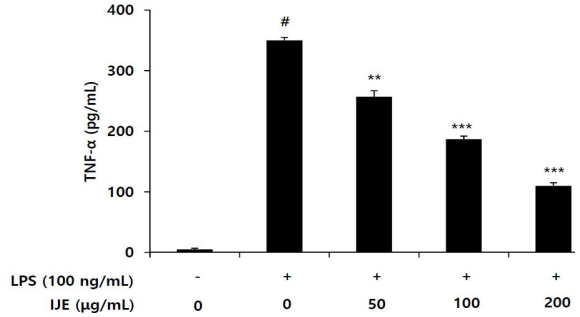

Figure 5: Effect of IJE on TNF-α production in activated BV-2 microglial cells. Suppression TNF-α expression by IJE was measured with ELISA test. BV-2 cells were treated with IJE at 0, 50, 100 and 200 µg/ml with or without 1 µg/ml of LPS for 4 h incubation. All data are presented for four independent experiments as the mean ± S.E.M. (n = 4); $^{\#}p < 0.001$, when compared with control; $^{**}p < 0.01$ and $^{***}p < 0.001$, when compared with LPS alone by Student t-test. (IJE: *Isodon japonicas extract*; TNF-α: Tumor necrosis factor-alpha; LPS: lipopolysaccharide)

It is well accepted that activated microglia can produce various neurotoxic substances, including nitric oxide synthesized by iNOS mRNA expression. To assess the effect of IJE on LPS-induced NO production in BV2 cells, Griess reagent was used. NO is a crucial messenger and releasing NO can play a role in the neuronal pathway for cell to cell communication, leading to neuroinflammation. In this study, it was observed that IJE significantly suppressed iNOS protein production in both LPS-stimulated BV-2 microglial cells in a dose-dependent fashion. Activated microglial cells are known to release a proinflammatory cytokines including TNF-α which may not only intensify the inflammatory cascade, but also causes the inflammatory harm [11,12]. Therefore, IJE had an effect on the production of TNF-α in LPS-activated BV-2 microglial cells. The results indicate that IJE significantly suppressed the production of TNF-α in the LPS-activated BV-2 cells. Considering the data obtained it was suggested that IJE might have a potent anti-neuroinflammatory activity via the inhibition of TNF-α, NO, as well as *i*NOS protein in activated microglia cell.

The major focus of this thesis is the microglial

response to neuroinflammation during disease. Therefore, the remaining parts of this introduction are devoted to microglial activation and immune function in the CNS. In addition, it propagates the release of inflammatory mediators responsible for the recruitment of additional neutrophils and macrophages in the CNS thereby hastening neuroinflammation [13]. Antioxidants and radical scavengers can reduce brain inflammation. DPPH free radical assay as one of the widely used methods for evaluating the free radical scavenging activities of several antioxidants [14]. *Isodon japonicas* is traditionally used in folk medicine to treat various ailments like tumors, gastrointestinal disorders, and inflammation.

Earlier reports revealed that *IJE* possess strong antioxidant compounds [8]. Previously, it had been demonstrated that *IJE* contains several phenolic compounds [9]. The antioxidant compounds such as kamebakaurin isolated from *Isodon japonicas* were well reported to have an anti-oxidant and anti-inflammatory activities [9]. In the present study, the IJE also exerted significant free radical scavenging effects, suggesting that IJE may contain anti-oxidant agents such as polyphenols.

CONCLUSION

The present study reports, for the first time, that IJE attenuates the neuro-inflammatory activity in activated murine BV-2 microglial cells. The anti-neuro-inflammatory effects of IJE might be attributed it its regulatory actions on the proinflammatory cytokine, TNF-α, as well as its strong antioxidant effects. Therefore, IJE can potentially be developed for the treatment of microglia-mediated neuroinflammatory disease.

DECLARATIONS

Competing interests

The authors declare that no conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors.

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