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원 저

Human placental extract suppresses lipopolysaccharide-induced expressions of cyclooxygenase-2 and inducible nitric oxide synthase in mouse BV2 microglial cells

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Abstract

Human placental extract (HPE), which is prepared from the placenta of healthy pregnant females, has been widely used in clinical field. HPE is known to possess anti-inflammatory, anti-viral, anti-oxidative, anti-mutagenic, and analgesic properties. In this study, the effect of HPE against lipopolysaccharide (LPS)-induced inflammation was investigated. From the present results, HPE was shown to suppress prostaglandin E2 synthesis (PGE2) and nitric oxide (NO) production by inhibition on the LPS-stimulated enhancement of the cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) expressions in mouse BV2 microglial cells. These results suggest that HPE may offer a valuable mean of therapy for the treatment of brain inflammatory diseases by attenuating LPS-induced PGE2 and NO production.

Key words : Human placental extr]haride, Prostaglandin E2 Nitric oxide

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Ⅰ. Introduction

Brain inflammation has been implicated in the pathogenesis of several types of neurodegenerative disease such as Alzheimer's disease (AD), Parkinson's disease, and ischemic brain injury[1-3]. Inflammation is a complex process induced by various chemical mediators that are released by both resident and infiltrating cells. Microglial cells are the resident macrophages and immune surveillance cells in the central nervous system (CNS), and they play a pivotal role in brain inflammatory, immune, and degenerative processes[4]. Activated microglial cells secrete numerous products including prostaglandins (PGs) and nitric oxide (NO)[5].

PGs are derived from arachidonic acid by the cyclooxygenase (COX) pathway, and they act as a key inflammatory mediator[5]. There are two isoforms of cyclooxygenase: cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2). While COX-1 is a constitutively expressed form involved in normal physiologic functions, COX-2 is expressed only in response to inflammatory signals such as cytokines and bacterial endotoxin lipopolysaccharide(LPS). COX-2 produces a large amount of PGE_2 and this induces the process of inflammation.

Nitric oxide (NO) is endogenously generated from L-arginine by nitric oxide synthase (NOS), and NO plays an important role in the regulation of many pathophysiological processes. NO that is secreted by activated microglia can rapidly react with O_2^- to produce the peroxynitrite anion[6]. Excessive NO and reactive oxygen species (ROS) production in the brain are known to contribute to neurodegenerative processes[7]. Several isoforms of NOS exist and these fall into three major classes: inducible NOS (iNOS), endothelial NOS (eNOS), and neuronal NOS (nNOS). Both nNOS and eNOS are constitutively expressed, whereas iNOS is inducible in response to immunologic activation and subsequent transcription[8]. iNOS is responsible for the overproduction of NO in inflammation[9].

Human placental extract (HPE) is prepared from the placenta of healthy pregnant females, and it is a rich resource forvarious bioactive substances such as polydeoxyribonucleotides (PDRN), RNA, DNA, peptides, amino acids, enzymes, and trace elements[10-11]. HPE has been reported to have various therapeutic effects including anti-inflammatory, anti-viral, anti-oxidative, anti-mutagenic, and analgesic properties[12-15].

Here in our present study, the effect of HPE on LPS-stimulated expressions of COX-1, COX-2, and iNOS, and on production of PGE_2 and NO in the mouse BV2 microglial cells was investigated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, reverse transcription-polymerase chain reaction (RT-PCR), PGE_2 immunoassay, and a NO detection kit.

Ⅱ. Materials and methods

1. Cell culture

The mouse BV2 microglial cells used in this experiment were cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco BRL, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) at 37 ℃ in 5% CO_2, 95% O_2 in a humidified cell incubator.

2. MTT assay for cell viability

Cell viability was determined by using the MTT assay kit (Boehringer Mannheim GmbH, Mannheim, Germany) as per the manufacturer's protocols. HPE solution was purchased from the Whasung Pharmacy Co. (Geochang, Korea). In order to determine the cytotoxicity of HPE, cells were treated with HPE at concentrations of 1 ㎍/㎖, 5 ㎍/㎖, 10 ㎍/㎖, 50 ㎍/㎖, and 100 ㎍/㎖ for 24 h, and cultures of the control group were left untreated. Ten ㎕ of the MTT labeling reagent was added to each well, and the plates were incubated for 4 h. Solubilization solution of 100 ㎕ was then added to each well, and the cells were incubated for another 12 h. The absorbance was then measured with a microtiter plate reader (Bio-Tek, Winooski, VT, USA) at a test wavelength of 595 ㎚ and a reference wavelength of 690 ㎚. The optical density (O.D.) was calculated as the difference between the absorbance at the reference wavelength and that at the test wavelength. The percent viability was calculated as (O.D. of drug-treated sample/control O.D.) x 100.

3. RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR)

To identify expressions of COX-1, COX-2, and iNOS mRNAs, RT-PCR was performed. The total RNA was isolated from BV2 microglial cells using RNAzol™B (TEL-TEST, Friendswood, TX, USA). Two ㎍ of RNA and 2 ㎕ of random hexamers (Promega, Madison, WI, USA) were added together, and the mixture was heated at 65℃ for 10 min. One ㎕ of AMV reverse transcriptase (Promega), 5 ㎕ of 10 mM dNTP (Promega), 1 ㎕ of RNasin (Promega), and 5 ㎕ of 10 x AMV RT buffer (Promega) were then added to the mixture, and the final volume was brought up to 50 ㎕ with diethyl pyrocarbonate (DEPC)-treated water. The reaction mixture was then incubated at 42 ℃ for 1 h.

PCR amplification was performed in a reaction volume of 40 ㎕ containing 1 ㎕ of the appropriate cDNA, 1 ㎕ of each set of primers at a concentration of 10 pM, 4 ㎕ of 10 x RT buffer, 1 ㎕ of 2.5 mM dNTP, and 2 units of Taq DNA polymerase (TaKaRa, Shiga, Japan). For mouse COX-1, the primer sequences were 5′-AGTGCGGTCCAACCT TATCC-3′ (a 20-mer sense oligonucleotide) and 5′-CCGCAGGTGATACTGTCGTT-3′ (a 20-mer anti-sense oligonucleotide). For mouse COX-2, the primer sequences were 5′-TGCATGTGGCTGTGGATGTCATCAA-3′ (a 25-mer sense oligonucleotide) and 5′-CACTAAGACAGACCCGTCATCTCCA-3′ (a 25-mer anti-sense oligonucleotide). For mouse iNOS, the primer sequences were 5′-GTGTTCCACCAGGAGATGTTG-3′(a 21-mer sense oligonucleotide) and 5′-CTCC TGCCCACTGAGTTCGTC-3′ (a 21-mer anti-sense oligonucleotide). For cyclophilin, the internal control used in the study, the primer sequences were 5′-ACCCCACCGTGTTCT TCGAC-3′ (a 20-mer sense oligonucleotide starting at position 52) and 5′-CATTTGCC ATGGACAAGATG-3′ (a 20-mer anti-sense oligonucleotide starting at position 332). The expected size of the PCR product was 382 bp for COX-1, 583 bp for COX-2, 500 bp for iNOS, and 299 bp for cyclophilin.

For COX-1, COX-2 and iNOS, the PCR procedure was carried out using a GeneAmp 9600 PCR system (Perkin Elmer, Norwalk, CT, USA) under the following conditions: an initial denaturation at 94℃ for 5 min, followed by 35 amplification cycles, each consisting of denaturation at 94℃ for 30 sec, annealing at 58℃ for 30 sec, and extension at 72℃ for 30

sec, with an additional extension step at the end of the procedure at 72℃ for 5 min. For cyclophilin, the PCR procedure was carried out under identical conditions except that 25 amplification cycles were executed. The final amount of RT-PCR product for each of the mRNA species was calculated densitometrically using Molecular Analyst™ version 1.4.1 (Bio-Rad, Hercules, CA, USA).

4. PGE_2 assay

The assessment of PGE_2 synthesis was performed using a commercially available PGE_2 competitive enzyme immunoassay kit (Amersham Pharmacia Biotech. Inc., Piscataway, NJ, USA). The cells were lysed, and then the cell lysates and standards were put into different wells on the goat anti-mouse IgG-coated microtiter plate provided in the kit. Mouse anti-PGE_2 antibody and peroxidase-conjugated PGE_2 were added to each well, and the plate was incubated and shaken at room temperature and for 1 h. The wells were drained, washed, and 3,3′,5,5′-tetramethylbenzidine/hydrogen peroxide solution was then added. The plate was incubated at room temperature with shaking, and the reaction was stopped after 30 min through the addition of H_2SO_4. The absorbance of the content of each well was then measured at 450 nm.

5. Measurement of NO generation

In order to determine the effect of HPE on NO synthesis, the amount of nitrite (NO_2^-) in cell-free culture supernatant was measured by using a commercially available NO detection kit (Intron, Inc., Seoul, Korea). After collection of 100 ㎕ of supernatant, 50 ㎕ N1 buffer was added to each well, and the plate was incubated at room temperature for 10 min. N2 buffer was then added and the plate was incubated at room temperature for 10 min. The absorbance of the content of each well was measured at 540 nm. The nitrite concentration was calculated from a nitrite standard curve.

6. Statistical analyses

The results are expressed as the mean and standard error mean (S.E.M.). The data were analyzed by one-way ANOVA followed by Duncan's post-hoc test using SPSS (version 11.5). Difference was considered statistically significant at $p<0.05$.

Ⅲ. Results

1. Effect of HPE on cell viability

The viability of cells incubated with HPE at 1 ㎍/㎖, 5 ㎍/㎖, 10 ㎍/㎖, 50 ㎍/㎖, and 100 ㎍/㎖ for 24 h were 95.64±2.32%, 95.35±1.65%,

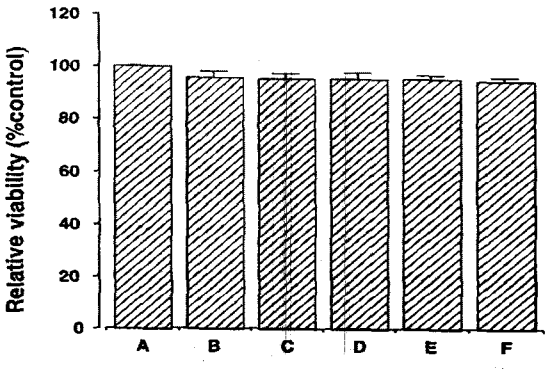

Fig. 1. Effect of human placental extract (HPE) on BV2 cell viability. Cell viability was determined via 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The results are presented as the mean ± standard error mean (S.E.M). A, Control; B, 1 ㎍/㎖ HPE-treated group; C, 5 ㎍/㎖ HPE-treated group; D, 10 ㎍/㎖ HPE-treated group; E, 50 ㎍/㎖ HPE-treated group; F, 100 ㎍/㎖ HPE-treated group.

95.25±2.05%, 95.28±1.49%, and 94.35±1.25% of the control value, respectively. The MTT assay revealed that HPE exerted no significant cytotoxicity in mouse BV2 microglial cells (Fig. 1).

2. Effect of HPE on the expressions of COX-1, COX-2, and iNOS m RNA

RT-PCR analysis of the mRNA levels of COX-1, COX-2 and iNOS was performed. In the present study, the mRNA level of COX-1, COX-2, and iNOS in the control cells was set as 1.00.

The level of COX-1 mRNA following treatment with 1 μg/mℓ LPS for 24 h was 0.99±0.06. It was decreased to 0.91±0.04, 0.73±0.01, and 0.45±0.04 in cells treated with 5 μg/mℓ HPE, 10 μg/mℓ HPE, and 50 μg/mℓ aspirin 1 h prior to the 1 μg/mℓ LPS exposure, respectively. LPS treatment exerted no significant effect on the COX-1 mRNA expression in mouse BV2 microglial cells. Pretreatment with 5 μg/mℓ HPE 1 h prior to the LPS exposure also exerted no significant effect on the COX-1 mRNA expression, however 10 μg/mℓ HPE and 50 μg/mℓ aspirin pretreatment 1 h prior to the 1 μg/mℓ LPS exposure significantly suppressed COX-1 mRNA expression.

The level of COX-2 mRNA following 1 μg/mℓ LPS treatment for 24 h was markedly increased to 33.94±1.80, but it was significantly decreased to 6.30±0.79, 4.28±0.95, and 1.56±0.24 in cells treated with 5 μg/mℓ HPE, 10 μg/mℓ HPE, and 50 μg/mℓ aspirin 1 h prior to the 1 μg/mℓ LPS exposure, respectively. LPS treatment significantly enhanced COX-2 mRNA expression in mouse BV2 microglial cells. Pretreatment with 5 μg/mℓ HPE, 10 μg/mℓ

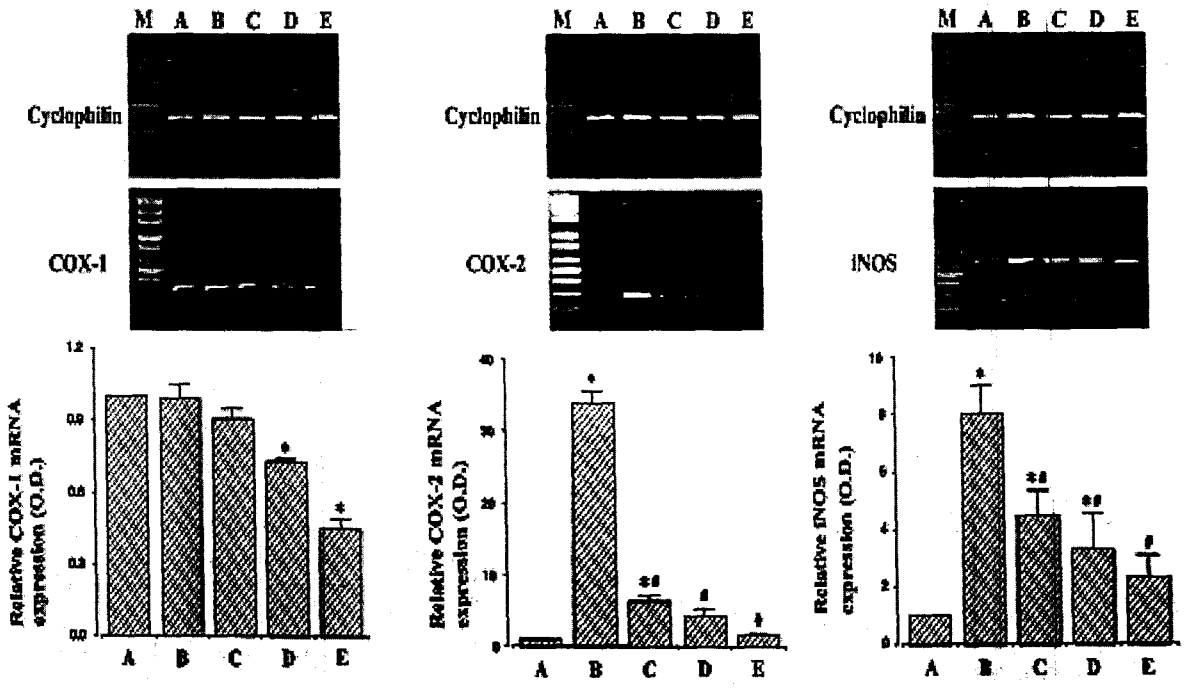

Fig. 2. Reverse transcription-polymerase chain reaction (RT-PCR) analysis of the mRNA levels of cyclooxygenase-1 (COX-1), cyclooxygenase-2 (COX-2), and inducible nitric oxide synthase (iNOS). Cyclophilin mRNA was used as the internal control. * represents $p<0.05$ compared to the control. # represents $p<0.05$ compared to the lipopolysaccharide (LPS)-treated group. M, Marker; A, Control; B, LPS-treated group; C, LPS- and 5 μg/mℓ human placental extract (HPE)-treated group; D, LPS- and 10 μg/mℓ HPE-treated group; E, LPS- and 50 μg/mℓ aspirin-treated group.

HPE, and 50 ㎍/㎖ aspirin suppressed LPS-induced COX-2 mRNA expression.

The level of iNOS mRNA was significantly increased to 8.05±2.97 following 1 ㎍/㎖ LPS treatment for 24 h, while it was decreased to 4.53±1.80, 3.31±1.24, and 2.33±0.78 in cells treated with 5 ㎍/㎖ HPE, 10 ㎍/㎖ HPE, and 50 ㎍/㎖ aspirin 1 h prior to the 1 ㎍/㎖ LPS exposure, respectively. LPS treatment significantly enhanced iNOS mRNA expression in mouse BV2 microglial cells. Pretreatment with 5 ㎍/㎖ HPE, 10 ㎍/㎖ HPE, and 50 ㎍/㎖ aspirin suppressed LPS-induced iNOS mRNA expression (Fig. 2).

3. Effect of HPE on PGE2 synthesis

From the results of the PGE_2 immunoassay, after 24 h of exposure to 1 ㎍/㎖ LPS, the amount of PGE_2 from the culture medium was increased from 35.50±0.86 pg/㎖ to 113.00±1.73 pg/㎖. It was decreased to 44.00±7.54 pg/㎖, 39.66±4.35 pg/㎖, and 34.66±1.52 by the treatment with 5 ㎍/㎖ HPE, 10 ㎍/㎖ HPE, and 50 ㎍/㎖ aspirin, respectively. LPS treatment increased PGE_2 synthesis in mouse BV2 microglial cells. Pretreatment with 5 ㎍/㎖ HPE, 10 ㎍/㎖ HPE, and 50 ㎍/㎖ aspirin significantly suppressed LPS-induced PGE_2 synthesis (Fig. 3).

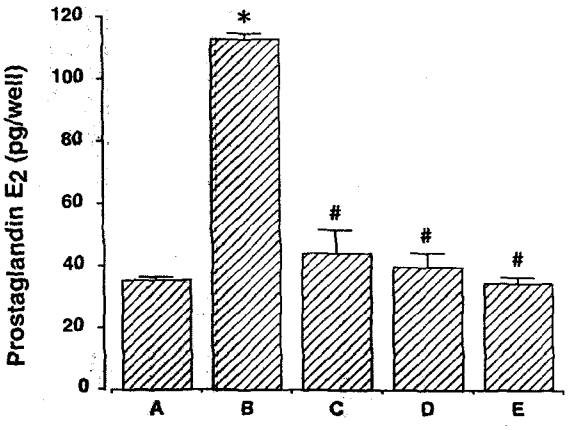

Fig. 3. Measurement of prostaglandin E2 in BV2 cells. * represents $p<0.05$ compared to the control. # represents $p<0.05$ compared to the lipopolysaccharide (LPS)-treated group. A, Control; B, LPS-treated group; C, LPS- and 5 ㎍/㎖ human placental extract (HPE)-treated group; D, LPS- and 10 ㎍/㎖ HPE-treated group; E, LPS- and 50 ㎍/㎖ aspirin-treated group.

4. Effect of HPE on NO synthesis

From the results of the NO detection assay, after 24 h of exposure to 1 ㎍/㎖ LPS, the amount of nitrite was increased from 10.60±0.40 μM to 22.95±1.45 μM. It was decreased to 19.30±0.60 μM, 13.05±0.56 μM, 12.52±0.19 μM, 11.27±1.11 μM, and 11.38±0.31 μM by treatment with 1 ㎍/㎖ HPE, 5 ㎍/㎖ HPE, 10 ㎍/㎖ HPE, 50 ㎍/㎖ HPE, and 50 ㎍/㎖ aspirin, respectively. LPS treatment increased NO synthesis in mouse BV2 microglial cells. Pretreatment with 5 ㎍/㎖ HPE, 10 ㎍/㎖ HPE, and 50 ㎍/㎖ aspirin significantly suppressed LPS-induced NO synthesis (Fig. 4).

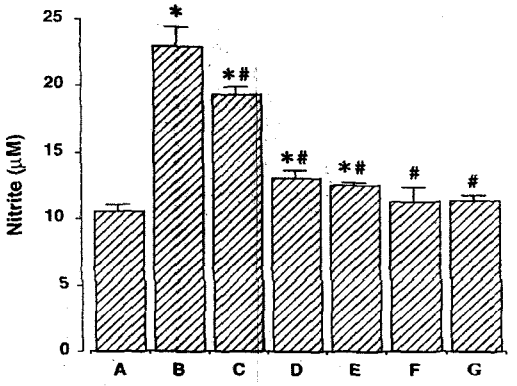

Fig. 4. Measurement of nitric oxide (NO) production in BV2 cells. * represents $p<0.05$ compared to the control. # represents $p<0.05$ compared to the lipopolysaccharide (LPS)-treated group. A, Control; B, LPS-treated group; C, LPS- and 1 ㎍/㎖ human placental extract (HPE)-treated group; D, LPS- and 5 ㎍/㎖ HPE-treated group; E, LPS- and 10 ㎍/㎖ HPE-treated group; F, LPS- and 50 ㎍/㎖ HPE-treated group; G, LPS- and 50 ㎍/㎖ aspirin-treated group.

5. Discussion

In the present study, HPE was shown to suppress PGE_2 and NO production through inhibiting LPS-stimulated expressions of the COX-2 and iNOS genes in the mouse BV2 microglial cells. It is well known that the human placenta has many biological and therapeutic components[11]. In many clinic studies, an aqueous extract of human placenta have been reported to possess anti-inflammatory, antiviral, anticytotoxic, and antioxidant properties[12-15]. Many studies have recently suggested that HPE is useful in suppressing inflammation induced by edema or granuloma in animal models[16].

Hominis Placenta is produced by drying human placenta extracted from the body of a healthy mother. It has been called as 胞衣[17], 混沌皮, 混元丹[18], 混沌衣[19], 佛架娑, 仙人衣, 混元母, 人胞, 人胎盤, 胎胞, 胎盤粉, 胎盤, 衣胞, 京河車, 臍河車, 河車[20] in many oriental medical books. The charcteristics of Hominis Placenta is known to be warm, sweet and salty[19,21]. The therapeutic actionis manifested in the Liver meridian, Spleen meridian, kidney meridian and Lung meridian[17,-18, 22-23]. It tonifies the Qi, nourishes the blood, tonifies the vital energy, and calms the spirits and settles the emotions[24-25]. Thus it is used on patients with blood deficiency, female deficiency syndrome, black skin symptoms, loss of weight due to abodominal illness, deficiency syndromes, consumptive diseases, epilepsy, stupor mental state, five kinds of consumptive disease and seven kinds of impairments, tidal fever due to steeming bone, cough, dumbness, lean body, deficiency of vital energy, hematemesis, epistaxis, back pain, infertility, impotence, and yellowish complexion[17-19, 26]. Hominis Placenta aqua-acupuncture tonifies reproductive functions[27], improves climacteric symptoms[28], cures diabetes mellitus[29], stalls cellular damage and enhances cellular recovery[30-32], improves peripheral facial palsy[33]. Also the effect of improving arthritis[34], osteoporosis[35-40], various pains are being reported[41]. Seo[30], Kang[39] and Woo[40] reports the effect of Hominis Placenta injection acupuncture inhibiting NO synthesis related with IL-1, TNF-α, INF-γcytokines, thus preventing cellular damages and progress of osteoporosis.

LPS is derived from the cell walls of gram-negative bacteria, and it mediates many of the inflammatory sequelae of infection[42]. LPS initiates a number of major cellular responses that play a vital role in the pathogenesis of inflammatory responses including the activation of inflammatory cells and the production of cytokines and other mediators.

In the present study, microglial cells activated by LPS produced a large amounts of PGs and NO, which are known be critical factors in determining the final outcome of a microglial reaction to pathological stimuli. PGs and NO synthesis are markedly increased when the inducible isoforms of COX and NOS are expressed.

It is also known that the COX-1 isozyme is a housekeeping protein in most tissues, and it catalyzes the synthesis of PGs used by the body for normal physiological functions. Thus, COX-1 is expressed at essentially a constant level, and this level does not fluctuate in response to various stimuli. In constant an inducible isoform, COX-2 expression is rapidly stimulated by tumor promoters, growth factors, cytokines, and pro-inflammatory molecules in variety of cell types[5]. COX-2 is known to be responsible for the production of the high levels of PGs in several pathological conditions such as inflammation, and it is the major

isoform expressed in inflammatory cells including microglia[43].

Nonsteroidal anti-inflammatory drugs (NSAIDs), such as aspirin, inhibit both isoforms of COX, and they exert both their beneficial effects (inhibition of COX-2) and potentially deleterious effects (inhibition of COX-1). The recent identification and better understanding of COX-1 and COX-2 has led to new approaches for the development of anti-inflammatory therapies. When we consider that NSAIDs constitute one of the largest groups of prescribed drugs worldwide, the search for selective inhibitors of COX-2 may well provide extensive clinical benefits in the future[44].

The present study shows that the expression of COX-2 mRNA in mouse BV2 microglial cells was significantly increased by LPS and that HPE and aspirin inhibited LPS-induced COX-2 mRNA expression in microglial cells. Aspirin and a high dose of HPE inhibited COX-1 mRNA expression, however, a low dose of HPE was not observed to inhibit COX-1 mRNA. The present results suggest that a low dose of HPE exerts its anti-inflammatory effect by COX-2 mRNA inhibition and this dose not incur the side effects such as peptic ulcer formation and renal dysfunction, which are sometimes associated with COX-1 inhibition.

NO production through the iNOS pathway is increased in inflammatory diseases, and excessive NO production induces cellular injury. Expression of the iNOS gene is increased in several pathophysiological conditions, and it produces large amounts of NO in response to inflammatory signals from such molecules as the cytokines and LPS[5,44]. The present study showed that iNOS mRNA expression in microglial cells was increased by LPS, and that HPE and aspirin inhibited the LPS-induced iNOS mRNA expression in microglial cells.

Here in our study, we have shown that HPE exerts its anti-inflammatory and analgesic effects probably by the suppression of COX-2 and iNOS mRNA expressions, and the final result is the inhibition of PGE_2 and NO synthesis. Based on our present results, it is possible that HPE can offer a valuable means of therapy for the treatment of brain inflammatory diseases by attenuating LPS-induced PGE_2 and NO synthesis.

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