Effects of the Aqueous Extract of *Rehmanniae Radix Preparata* on Lipopolysaccharide-induced Expressions of Cyclooxygenase-2 and Inducible Nitric Oxide Synthase in Mouse BV2 Microglial Cells

Chang Young Jung, Yun Hee Sung[1], Sung Eun Kim[1], Chang Ju Kim[1], Seung Ho Han[2], Choong Yeol Lee*

Department of Physiology, College of Oriental Medicine, Kyungwon University,
1: Department of Physiology, College of Medicine, Kyung Hee University, 2: Department of Physiology, College of Medicine, Eulji University

Rehmanniae radix preparata is the root of Rehmanniae glutinosa Liboschitz var. purpurea Makino which has been classified into Scrophulariaceae. Rehmanniae radix preparata has been used for the treatment of diabetes, for the relief of the pain, and for the anti-oxidative action. In this study, the effect of the aqueous extract of Rehmanniae radix preparata on lipopolysaccharide-induced inflammation was investigated by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, reverse transcription-polymerase chain reaction (RT-PCR), Western blot, prostaglandin E2 immunoassay, and nitric oxide (NO) detection in mouse BV2 microglial cells. In the present results, the aqueous extract of Rehmanniae radix preparata suppressed prostaglandin E2 (PGE2) synthesis and nitric oxide production by inhibiting the lipopolysaccharide-stimulated expressions of cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) mRNA and protein in mouse BV2 cells. These results show that Rehmanniae radix preparata exerts anti-inflammatory effect probably by suppressing of COX-2 and iNOS expressions.

Key words : Rehmanniae radix preparata, lipopolysaccharide, cyclooxygenase-2, prostaglandin E2, nitric oxide

Introduction

Microglia are immune cells in the central nervous system (CNS), and they are activated by brain injuries such as ischemia, trauma, infection, and other neurodegenerative diseases[1,2]. The activated microglia release various bioactive molecules including nitric oxide (NO), reactive oxygen species (ROS), and cytokines such as tumor necrosis factor-α (TNF-α)[3]. Lipopolysaccharide (LPS) activates microglia and plays vital roles in the pathogenesis of inflammatory responses[4].

As a key inflammatory mediator, prostaglandin E2 (PGE2) is converted from arachidonic acid by cyclooxygenase (COX). There are two isoforms of COX: COX-1 and COX-2. While COX-1 is a constitutively expressed form in normal physiologic functions, COX-2 is expressed only in response to inflammatory signals such as cytokines and bacterial endotoxin, LPS. COX-2 produces large amount of PGE2 that induces inflammation[5,6].

* To whom correspondence should be addressed at : Choong Yeol Lee, Department of Physiology, College of Oriental Medicine, Kyungwon University, 65 San, Bokjeong-dong, Sujeong-gu, Seongnam, Kyunggi-do, Korea
· E-mail : cylee@kyungwon.ac.kr, · Tel : 031-750-5419
· Received : 2006/06/29 · Revised : 2006/07/14 · Accepted : 2006/08/05

As a neuromodulator in the CNS, NO is an important physiological messenger and effector molecule in many biological system, including immunological, neuronal and cardiovascular tissues[7]. NO is endogenously generated from L-arginine by nitric oxide synthase (NOS)[8]. Three types of NOS have been identified: endothelial NOS (eNOS), inducible NOS (iNOS), and neuronal NOS (nNOS)[9].

Rehmanniae radix preparata is the root of Rehmanniae glutinosa Liboschitz var. purpurea Makino which has been classified into Scrophulariaceae. Rehmanniae radix preparata consists of saccharides, catalpol, vitamin A, arginine, mannitol, and β-sitosterol. Rehmanniae radix preparata has been used for the treatment of diabetes, for the relief of pain, for the anti-oxidative action, and for the anti-inflammatory effect[10-12].

In the present study, the effects of the aqueous extract of Rehmanniae radix preparata on the LPS-stimulated expressions of COX-2 and iNOS in mouse BV2 microglial cells were investigated. The effects of the aqueous extract of Rehmanniae radix preparata on PGE2 synthesis and NO production were also evaluated. For this study, 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay, reverse transcription-polymerase chain reaction (RT-PCR), Western blot, PGE2 immunoassay, and NO detection were performed.

Materials and Methods

1. Cell culture

Mouse BV2 microglial cells were cultured in Dulbecco's modified Eagle's Medium (DMEM; Gibco BRL, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco BRL) at 37°C in 5% CO_2-95% O_2 in a humidified cell incubator. The cells were plated onto culture dishes at a density of 2 x 10^4 cells/cm^2 at 24 h prior to drug treatment.

2. Preparation of the aqueous extract of Rehmanniae radix preparata

To obtain the aqueous extract of Rehmanniae radix preparata, 50 g of Rehmanniae radix preparata was added to distilled water, and extraction was performed by heating at 80°C for 2 h, concentrating with rotary evaporator, and lyophilizing. The resulting powder, weighing 28.48 g (a yield of 56.96%), was diluted to the concentrations needed with autoclaved distilled water and filtered through a 0.22 μm syringe filter before use.

3. MTT cytotoxicity assay

Mouse BV2 microglial cells were grown in a final volume of 100 μl culture medium per well in a 96-well plates. In order to determine the cytotoxicity of Rehmanniae radix preparata, the cells were treated with the aqueous extract of Rehmanniae radix preparata at concentrations of 10 μg/ml, 50 μg/ml, 100 μg/ml, 500 μg/ml, 1,000 μg/ml, 5,000 μg/ml, and 10,000 μg/ml for 24 h. The cells in the control group were left untreated. After adding 10 μl of the MTT labeling reagent containing 5 mg/ml 3-(4,5-dimethyl thiazol-2yl)-2,5-diphenyl tetrazolium bromide in phosphate-buffered saline to each well, the plates were incubated for 4 h. Solubilization solution 100 μl containing 10% sodium dodecyl sulfate in 0.01 M hydrochloric acid was 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 nm with a reference wavelength of 690 nm. The optical density (O.D.) was calculated as the difference between the absorbance at the reference wavelength and that observed at the test wavelength. Percent viability was calculated as (O.D. of drug-treated sample/control O.D.) x 100.

4. RNA isolation and RT-PCR

To identify the expressions of COX-2 and iNOS mRNA, RT-PCR was performed. The total RNA was isolated from Mouse BV2 microglial cells using RNAzolTMB (TEL-TEST, Friendswood, TX, USA). Two μg of RNA and 2 μl of random hexamers (Promega, Madison, WI, USA) were added together, and the mixture was heated at 65°C for 10 min. one μl of AMV reverse transcriptase (Promega), 5 μl of 2.5 mM dNTP (Promega), 1 μl of RNasin (Promega), and 8 μl of 5 x AMV RT buffer (Promega) were then added to the mixture, and the final volume was brought up to 40 μl volume with diethylpyrocarbonate (DEPC)-treated water. The reaction mixture was then incubated at 42°C for 2 h.

PCR amplification was performed in a reaction volume of 40 μl containing 2 μl of the appropriate cDNA, 0.3 μl of each set of primers at a concentration of 10 pM, 4 μl of 10 x RT buffer, 1 μl of 2.5 mM dNTP, and 0.3 units of Taq DNA polymerase (Takara, Shiga, Japan). For mouse COX-2, the primer sequences were 5'-GAACATTGTGAACAACATCCC-3' (a 21-mer sense oligonucleotide) and 5'-GGTGGCATACATCATCAGACC-3' (a 21-mer anti-sense oligonucleotide). For mouse iNOS, the primer sequences were 5'-CAAGAGTTTGACCAGAGGACC-3' (a 21-mer sense oligonucleotide) and 5'-TGGAACCACTCCTACTTGGGA-3' (a 21-mer anti-sense oligonucleotide). For GAPDH, the internal control used in the study, the primer sequences were 5'-TGGTGCTGAGTATGTCGTCC-3' (a 20-mer sense oligonucleotide) and 5'-TTGTCATTGAGAGCAATGCC-3' (a 20-mer anti-sense oligonucleotide). The expected size of the PCR product was 630 bp for COX-2, 160 bp for iNOS, and 650 bp for GAPDH.

For COX-2 and iNOS, the PCR procedures were carried out using a PTC-0150 MiniCycler (Bio-Rad, Hercules, CA, U.S.A.) under the following conditions: initial denaturation at 94°C for 5 min, followed by 35 amplification cycles, each consisting of denaturation at 94°C for 45 sec, annealing at 60°C for 45 sec, and extension at 72°C for 60 sec, with an additional extension step at the end of the procedure at 72°C for 10 min. For GAPDH, the PCR procedure was under the following conditions: initial denaturation at 94°C for 5 min, followed by 30 amplification cycles, each consisting of denaturation at 94°C for 30 sec, annealing at 58°C, and extension at 72°C for 45 sec, with an additional extension step at the end of the procedure at 72°C for 10 min. The final amount of RT-PCR product for each of the mRNA species was calculated densitometrically using Molecular AnalystTM version 1.4.1 (Bio-Rad, Hercules, CA, USA).

5. Western blot analysis

Mouse BV2 microglial cells were lysed in a ice-cold whole cell lysate buffer containing 50 mM HEPES (pH 7.5), 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM magnesium chloride hexahydrate, 1 mM ethyleneglycol-bis-(β-aminoethyl

ether)-N,N'-tetraacetic acid (EGTA), 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM sodium ortho vanadate, and 100 mM sodium floride, and the mixture was incubated 30 min at 4°C. Cell debris was removed by microcentrifugation, followed by quick freezing of the supernatant. The protein concentration was measured using a Bio-Rad colorimetric protein assay kit (Bio-Rad, Hercules, CA, USA). Protein of 40 μg was separated on SDS-polyacrylamide gels and transferred onto a nitrocellulose membrane (Schleicher & Schuell GmbH, Dassel, Germany). Goat COX-2 antibody (1:2000; Santa Cruz Biotech, CA, USA), and rabbit iNOS antibody (1:500; Santa Cruz Biotech) were used as a primary antibody. Horseradish peroxidase-conjugated anti-goat antibody (1:2000; Santa Cruz Biotech) was used to probe for COX-2, and anti-rabbit antibody (1:4000; Santa Cruz Biotech) for iNOS wasused as a secondary antibody.Band detection was performed using the enhanced chemiluminescence (ECL) detection system (Amersham Pharmacia Biothech GmbH, Freiburg, Germany).

6. Measurement of PGE2 synthesis

Assessment of PGE2 synthesis was performed using a commercially available PGE2 competitive enzyme immunoassay kit (Amersham Pharmacia Biotech. Inc., Piscataway, NJ, USA). Supernatant of 100 μl from the culture medium and the standards were put into different wells on the goat anti-mouse IgG-coated microtiter plate provided in the kit. Mouse anti-PGE2 antibody and peroxidase-conjugated PGE2 were added to each well, and the plate was incubated at room temperature with shaking for 2 h. The wells were drained and washed, and 3,3',5,5'-tetramethylbenzidine/hydrogen peroxide solution was then added. The plate was incubated at room temperature with shaking for 30 min. The absorbance of the content of each well was then measured at a wavelength of 630 nm.

7. Determination of NO production

In order to determine the effect of the aqueous extract of Rehmanniae radix preparata on NO production, the amount of nitrite in the supernatant was measured, based on the Griess reaction, as an indicator of NO production. After collection of 100 μl of cell culture medium, 50 μl of 1% sulfanilamide was added to each well, and the plate was incubated at room temperature for 10 min. Naphtylethylenediamine of 0.1% containing 5% phosphoric acid 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 a wavelength of 540 nm. The nitrite concentration was calculated from a nitrite standard curve generated by mixing 0 to 200 μM sodium nitrite solutions with the Griess reagent. The standard curve was typically linear between 0 and 200 μM of sodium nitrite.

8. Statistical analysis

The results are presented as the mean ± standard error of the mean (S.E.M.). The data were analyzed by one-way ANOVA followed by Duncan's post-hoc test using SPSS. The differences were considered statistically significant for $P < 0.05$.

Results

1. Effect of the aqueous extract of Rehmanniae radix preparata on viability of mouse BV2 microglial cells

In order to assess the cytotoxic effect of the aqueous extract of Rehmanniae radix preparata on the mouse BV2 microglial cells, the cells were cultured with Rehmanniae radix preparata at final concentrations of 10 μg/ml, 50 μg/ml, 100 μg/ml, 500 μg/ml, 1,000 μg/ml, 5,000 μg/ml, and 10,000 μg/ml for 24 h, and MTT assays was then carried out. The cells cultured in Rehmanniae radix preparata-free media was used as the control. The viability of cells incubated with Rehmanniae radix preparata at concentrations of 10 μg/ml, 50 μg/ml, 100 μg/ml, 500 μg/ml, 1,000 μg/ml, 5,000 μg/ml, and 10,000 μg/ml for 24 h was 99.41 ± 1.70%, 102.40 ± 1.51%, 104.48 ± 2.52%, 105.93 ± 2.54%, 104.28 ± 3.21%, 105.97 ± 1.76%, and 107.52 ± 2.73% of the control value, respectively (Fig. 1).

The present results show that Rehmanniae radix preparata extract exerted no significant cytotoxicity until it was at a concentration of 10,000 μg/ml.

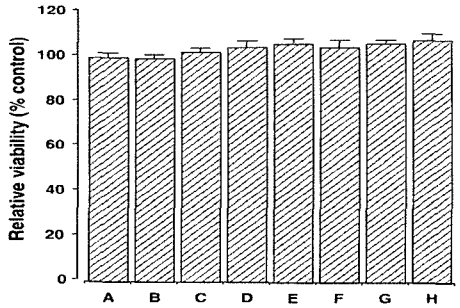

Fig. 1. Effect of Rehmanniae radix preparata on cell viability. Viability was determined via the MTT assay. (A) Control group; (B) 10 μg/ml Rehmanniae radix preparata-treated group; (C) 50 μg/ml Rehmanniae radix preparata-treated group; (D) 100 μg/ml Rehmanniae radix preparata-treated group; (E) 500 μg/ml Rehmanniae radix preparata-treated group; (F) 1,000 μg/ml Rehmanniae radix preparata-treated group; (G) 5,000 μg/ml Rehmanniae radix preparata-treated group; (H) 10,000 μg/ml Rehmanniae radix preparata-treated group. The results are presented as mean ± standard error mean (S.E.M.).

2. Effect of the aqueous extract of Rehmanniae radix preparata on mRNA expressions of COX-2 and iNOS

RT-PCR analysis of the mRNA level of COX-2 and iNOS was performed to estimate the relative level of expressions of

these genes. In the present study, the mRNA levels of COX-2 and iNOS in the control cells were set as 1.00.

The level of COX-2 mRNA was markedly increased to 4.99 ± 0.18 following a treatment with 2 μg/ml LPS for 24 h. The level of COX-2 mRNA was decreased to 3.93 ± 0.21, 3.53 ± 0.30, and 3.48 ± 0.44 in the cells pre-treated with the aqueous extract of Rehmanniae radix preparata at 500 μg/ml, 1,000 μg/ml, and 200 μM acetylsalicylic acid (ASA), respectively one hour before LPS exposure.

The level of iNOS mRNA following a treatment with 2 μg/ml LPS for 24 h was markedly increased to 2.61 ± 1.77. The level of iNOS mRNA was decreased to 1.91 ± 0.13, 1.38 ± 0.10, and 1.06 ± 0.03 in the cells pre-treated with the aqueous extract of Rehmanniae radix preparata at 500 μg/ml, 1,000 μg/ml, and 200 μM acetylsalicylic acid (ASA), respectively one hour before LPS exposure (Fig. 2).

The present results show that LPS enhanced COX-2 and iNOS mRNA expressions in BV2 microglial cells and that the aqueous extract of Rehmanniae radix preparata suppressed LPS-induced COX-2 and iNOS mRNA expressions.

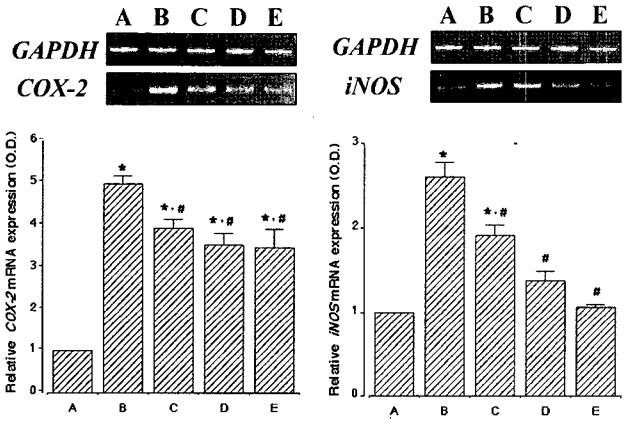

Fig. 2. Results of reverse transcription-polymerase chain reaction (RT-PCR) analysis of the mRNA levels of cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS). Cells at a density of 2 x 10^5 were pre-treated with Rehmanniae radix preparata at concentrations of 500 μg/ml, and 1,000 μg/ml for one hour and then treated with 2 μg/ml LPS for 24 hours. GAPDH mRNA was used as the internal control. * P <0.05 compared to the control. # P <0.05 compared to the LPS-treated group. (A) Control; (B) LPS-treated group; (C) LPS-treated group and 500 μg/ml Rehmanniae radix preparata-pre-treated group; (D) LPS-treated group and 1,000 μg/ml Rehmanniae radix preparata-pre-treated group; (E) LPS-treated group and 200 μM acetylsalicylic acid (ASA)-pre-treated group.

3. Western blot analysis of COX-2 and iNOS protein

Treatment with 2 μg/ml LPS for 24 h increased the level of COX-2 protein (72 kDa) and iNOS protein (130 kDa) in mouse BV2 microglial cells. Treatment with Rehmanniae radix preparata at 500 μg/ml, 1,000 μg/ml, and 200 μM ASA however, decreased the LPS-induced expressionsof COX-2 protein and iNOS protein (Fig. 3).

The present results show that LPS enhanced COX-2 and iNOS protein expressions in BV2 cells and Rehmanniae radix preparata extract suppressed LPS-induced COX-2 protein and iNOS protein expression.

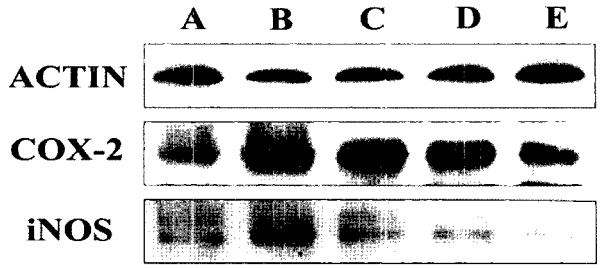

Fig. 3. Results of Western blot analysis of the protein levels of cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS). Cells at a density of 2 x 10^4 were pre-treated with Rehmanniae radix preparata at concentrations of 500 μg/ml, and 1,000 μg/ml, treated with 2 μg/ml LPS for 24 h. Actin was used as the internal control (46 kDa). * P <0.05 compared to the control. # P <0.05 compared to the LPS-treated group. (A) Control; (B) LPS-treated group; (C) LPS-treated group and 500 μg/ml Rehmanniae radix preparata-pre-treated group; (D) LPS-treated group and 1,000 μg/ml Rehmanniae radix preparata-pre-treated group; (E) LPS-treated group and 200 μM acetylsalicylic acid (ASA)-pre-treated group.

4. Effect of the aqueous extract of Rehmanniae radix preparata on PGE2 synthesis

From PGE2 immunoassay, the amount of PGE2 from the culture medium was increased from 141.50 ± 24.71 pg/ml to 522.74 ± 12.13 pg/ml after 24 h of exposure to LPS. PGE2 synthesis was decreased to 265.11 ± 20.51 pg/ml, 193.16 ± 48.40 pg/ml, and 182.29 ± 59.34 pg/ml by the pre-treatment with the aqueous extract of Rehmanniae radix preparata one hour before LPS exposure at 500 μg/ml, 1,000 μg/ml, and 200 μM ASA, respectively (Fig. 4).

The present results show that LPS enhanced PGE2 synthesis in BV2 cells and that the aqueous extract of Rehmanniae radix preparata suppressed LPS-induced PGE2 synthesis.

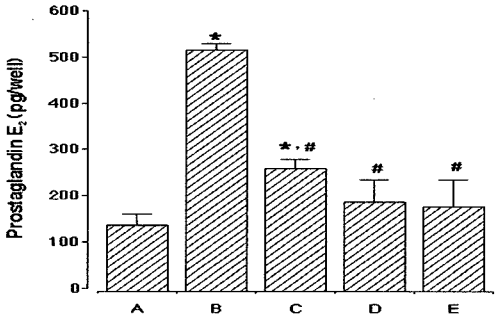

Fig. 4. Measurement of prostaglandin E2 (PGE2) synthesis in mouse BV2 microglial cells. Cells at a density of 2 x 10^4 were pre-treated with Rehmanniae radix preparata at concentrations of 500 μg/ml and 1,000 μg/ml for one hour and then treated with 2 μg/ml LPS for 24 h. * P <0.05 compared to the control. # P <0.05 compared to the LPS-treated group. (A) Control; (B) LPS-treated group; (C) LPS-treated group and 500 μg/ml Rehmanniae radix preparata-pre-treated group; (D) LPS-treated group and 1,000 μg/ml Rehmanniae radix preparata-pre-treated group; (E) LPS-treated group and 200 μM acetylsalicylic acid (ASA)-pre-treated group.

5. Effect of the aqueous extract of Rehmanniae radix preparata on NO production

From the NO detection assay, the amount of nitrite was increased from 11.75 ± 0.25 μM to 49.05 ± 1.96 μM after 24 h of exposure to LPS. NO synthesis was decreased to 40.52 ±

2.49 μM, 30.20 ± 1.05 μM, and 28.97 ± 3.90 μM by treatment with the aqueous extract of Rehmanniae radix preparata at 500 μg/ml, 1,000 μg/ml, and 200 μM ASA, respectively (Fig. 5).

The present results show that LPS enhanced NO production in BV2 cells and that the aqueous extract of Rehmanniae radix preparata suppressed LPS-induced NO production.

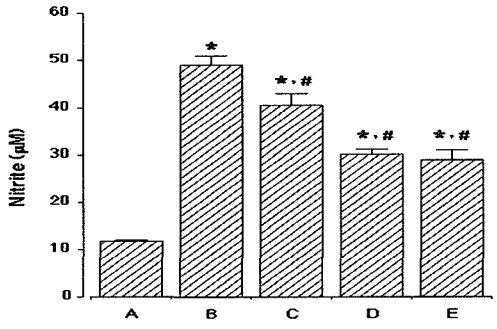

Fig. 5. Measurement of nitric oxide (NO) production in mouse BV2 microglial cells. Cells at a density of 2 x 10^4 were pre-treated with Rehmanniae radix preparata at concentrations of 500 μg/ml, and 1,000 μg/ml, treated with 2 μg/ml LPS for 24 h. * P <0.05 compared to the control, # P <0.05 compared to the LPS-treated group. (A) Control; (B) LPS-treated group; (C) LPS-treated group and 500 μg/ml Rehmanniae radix preparata-pre-treated group; (D) LPS-treated group and 1,000 μg/ml Rehmanniae radix preparata-pre-treated group; (E) LPS-treated group and 200 μM acetylsalicylic acid (ASA)-pre-treated group.

Discussion

In the present results, LPS enhanced COX-2 and iNOS expressions in mouse BV2 microglial cells. LPS activates inflammatory mediators and plays vital roles in the pathogenesis of inflammatory responses[4].

Inflammation is a complex process which commences with a primary reaction in tissues. Pain is one of the signs of inflammation, and it is important clues of immunological activity[13]. Pain is generated by the activation of astrocytes and microglia which release neurotransmitters including substance P, glutamate, and fractalkine. These neurotransmitters are reported to excite the pain-responsive neurons, resulting in the production of ROS, NO, prostaglandins, and growth factors[14].

In the present results, the aqueous extract of Rehmanniae radix preparata inhibited LPS-stimulated enhancement of COX-2 enzyme activity and PGE2 production in the mouse BV2 microglial cells. Prostaglandins exert their potent biological effects by binding to specific cell-surface receptors[15]. Also, prostaglandins sensitize pain receptors in both central and peripheral neurons[16]. As a major metabolite of the COX-2 pathway, PGE2 has emerged as an important lipid mediator of inflammatory and immune regulatory processes. PGE2 has been implicated in the pathogenesis of acute and chronic inflammatory disease states[17]. One of enzyme that is particulary important in inflammation is COX. COX is the first enzyme in the pathway by which arachidonic acid is converted to prostaglandins and thromboxanes. Specific COX-2 inhibitors are also known to attenuate the symptoms of inflammation[9].

In the present results, the aqueous extract of Rehmanniae radix preparata inhibited LPS-stimulated enhancement of iNOS enzyme activity and NO production in the mouse BV2 microglial cells. NO modulates the activity of COX-2 in a cGMP-independent manner, and plays a critical role in the release of PGE2 by direct activation of COX-2[18]. NO produced by NOS is a key regulator of homeostasis[19], however, over-production of NO occurs in inflammatory condition after the induction and expression of iNOS. iNOS is induced in response to agents such as interleukin 1β, TNF-α, interferon-γ, LPS. The agents that inhibit activity or induction of iNOS may be a useful tool with therapeutic focus in many inflammatory processes[20].

Rehmanniae radix preparata has anti-bacterial and anti-inflammatory effects. It has been used for the treatment of hectic fever, night sweat, and dizziness[12]. As the major active constituent of Rehmanniae radix preparata, β-sitosterol is known to have uterotrophic effect, to exert anti-viral, anti-inflammatory, and anti-febrile effects[21]. Based on the present results, the suppressive effect of Rehmanniae radix preparata on COX-2 and iNOS can be ascribed to the action of β-sitosterol.

Our present study has shown that Rehmanniae radix preparata exerts anti-inflammatory and analgesic effects probably by suppressing COX-2 and iNOS expressions, and resulting in the inhibition of PGE2 synthesis and NO production.

Acknowledgements

This study was supported by a grant of the Oriental Medicine R&D Project, Ministry of Health & Welfare, Republic of Korea. (0405-OD00-0815-B050049)

References

1. Gonzalez-Scarano, F., Baltuch, G. Microglia as mediators of inflammatory and degenerative diseases. Annu Rev Neurosci 22, 219-240, 1999.
2. Min, K.J., Yang, M.S., Jou, I., Joe, E.H. Protein kinase A mediates microglial activation induced by plasminogen and gangliosides. Exp Mol Med 36, 461-467, 2004.
3. Kim, W.K., Hwang, S.Y., Oh, E. S., Piao, H.Z., Kim, K.W., Han, I.O. TGF-α represses activation and resultant death of microglia via inhibition of phosphatidylinositol 3-kinase activity. J Immunol 172, 7015-7023, 2004.
4. Kubes, P., McCafferty, D.M. Nitric oxide and intestinal inflammation. Am J Med 109, 150-158, 2000.

5. Mitchell, J.A., Larkin, S., Williams, T.J. Cyclooxygenase-2: regulation and relevance in inflammation. Biochem Pharmacol 50, 1535-1542, 1995.
6. Crofford, L.J., Lipsky, P.E., Brooks, P., Abramson, S.B., Simon, L.S., Van de Putte, L.B. Basic biology and clinical application of specific cyclooxygenase-2 inhibitors. Arthritis Rheum 43, 4-13, 2000.
7. Bredt, D.S., Snyder, S.H. Nitric Oxide: a physiologic messenger molecule. Annu Rev Biochem 63, 175-195, 1994.
8. Dawson, T.M., Dawson, V.L., Snyder, S.H. A novel neuronal messenger molecule in brain: the free radical, nitric oxide. Ann Neurol 32, 297-311, 1992.
9. Yui, Y., Hattori, R., Kosuga, K., Eizawa, H., Hiki, K., Kawai, C. Purification of nitric oxide synthase from rat macrophages. J Biol Chem 266, 12544-12547, 1991.
10. Jeong, H.J., Kim, I.H. Comparative studies on the antidiabetic activities of Rehmannia Radices. Chung-Ang J Pharm Sci 4, 22-31, 1990.
11. Jo, S.I. Anti oxidative effects of RADIX REHMANNIAE PREPARATA on toxic agent induced kidney cell injury. Kor J Herbology 18, 119-126, 2003.
12. Kim, H.M., An, C.S., Jung, K.Y., Choo, Y.K., Park, J.K., Nam, S.Y. Rehmannia glutinosa inhibits tumor necrosis factor-alpha and interleukin-1 secretion from mouse astrocytes. Pharmacol Res 40, 171-176, 1999.
13. McGeer, P.L., McGeer, E.G. The inflammatory response system of brain: implications for therapy of Alzheimer and other neurodegenerative diseases. Brain Res Brain Res Rev 21, 195-218, 1995.
14. Watkins, L.R., Maier, S.F. The pain of being sick: implications of immune-to-brain communication for understanding pain. Annu Rev Psychol 51, 29-57, 2000.
15. Thierauch, K.H., Dinter, H., Stock, G. Prostaglandins and their receptors: Ⅱ. Receptor structure and signal transduction. J Hypertens 12, 1-5, 1994.
16. Mitchell, J.A., Larkin, S., Williams, T.J. Cyclooxygenase-2: regulation and relevance in inflammation. Biochem Pharmacol 50, 1535-1542, 1995.
17. Hinz, B., Brune, K., Pahl, A. Prostaglandin E(2) upregulates cyclooxygenase-2 expression in lipopolysaccharide-stimulated RAW 264.7 macrophages. Biochem Biophys Res Commun 272, 744-748, 2000.
18. Salvemini, D., Misko, T.P., Masferrer, J. L., Seibert, K., Currie, M.G., Needleman, P. Nitric oxide activates cyclooxygenase enzyme. Proc Natl Acad Sci USA 90, 7240-7244, 1993.
19. Vane, J.R., Mitchell, J.A., Appleton, I., Tomlinson, A., Bishop-Bailey, D., Croxtall, J., Willoughby, D.A. Inducible isoforms of cyclooxygenase and nitric-oxide synthase in inflammation. Proc Natl Acad Sci USA 91, 2046-2050, 1994.
20. Szabo, C. Alternations in nitric oxide production in various forms of circulatory shock. New Horiz 3, 2-32, 1995.
21. Hyun, J.W., Yang, Y.M., Sung, M.S., Chung, H.S., Paik, W.H., Kang, S.S., Park, J.G. The cytotoxic activity of sterol derivatives from Pulsatilla chinensis Regal. J Korean Cancer 28, 145-150, 1996.