Full Paper

***Panax notoginseng* Attenuates the Infarct Volume in Rat Ischemic Brain and the Inflammatory Response of Microglia**Hye Young Son^{1,†}, Hyung Soo Han^{2,†}, Hyo Won Jung¹, and Yong-Ki Park^{1,*}¹*Oriental Medicine Drug R&D Center and Oriental Medicine Research Institute, College of Oriental Medicine, Dongguk University, Gyeongju 780-714, South Korea*²*Department of Physiology, School of Medicine, Kyungpook National University, Daegu 700-422, South Korea*

Received July 29, 2008; Accepted December 1, 2008

Abstract. The roots of *Panax notoginseng* (PN) are commonly used as a therapeutic agent to stop hemorrhage and as a tonic to promote health in traditional Korean medicine. Stroke triggers an inflammatory response that not only plays a central role in the pathogenesis of cerebral ischemia, but also induces secondary damage. This study was designed to investigate the neuroprotective effects of the methanol extract of PN on the infarct volume induced by middle cerebral artery occlusion (MCAO) (90-min occlusion and 24-h reperfusion) in rat brains. The PN extract (50 mg/kg, i.p.) was administered 2 h after the onset of MCAO. The PN-treated groups had a reduction in infarct volume by $23.82 \pm 8.9\%$. In the PN extract-treated groups, the microglial density was significantly decreased in the peri-infarct region; the underlying mechanism was inhibition of inflammatory mediators, such as inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX)-2, via blocking of the NF- κ B pathway. Furthermore, in vitro studies showed that the PN extract significantly reduced the production of iNOS-derived NO and COX-2-derived prostaglandin E₂ through the regulation of gene transcription levels in primary microglia and BV-2 cells. These results suggest that anti-inflammatory and microglial activation inhibitory effects of the PN extract may contribute to its neuroprotective effects in brain ischemia.

Keywords: inflammation, microglia, *Panax notoginseng*, stroke**Introduction**

Acute ischemic stroke is the leading cause of adult disability and it is an important cause of death in industrialized countries, with an incidence affecting up to 0.2% of the population every year (1). Stroke triggers an inflammatory reaction that progresses for hours after the onset of a stroke; inflammation plays a central role in the pathogenesis of ischemic stroke. Inflammation is thought to contribute to the genesis of secondary damage as the consequence of microglial activation, resident perivascular and parenchymal macrophages, and infiltration of peripheral inflammatory cells. Therefore, anti-inflammatory treatment may reduce ischemic brain injury and enhance stroke recovery.

Microglia, the resident brain macrophages of the brain, function as scavenger cells in the event of infection, inflammation, trauma, ischemia, and neurodegeneration in the central nervous system (CNS) (2, 3). Once activated, microglia can undergo morphologic transformation into phagocytes, making them virtually indistinguishable from circulating macrophages (4). Studies have shown that inflammatory cells infiltrate around the ischemic area (5); and several proinflammatory genes or mediators, such as inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), and proinflammatory cytokines, are strongly expressed in the ischemic brain (6). In transient middle cerebral artery occlusion (MCAO) in rats, phagocytic microglia have been documented in the cerebral cortex of the ischemic hemisphere (7, 8).

Cerebral ischemia enhances iNOS expression in neurons, endothelial cells, and microglia (9, 10). That is, iNOS clearly plays a role in stroke outcome, as evidenced by its selective inhibition or its genetic deletion in rats

[†]These two authors equally contribute to this work.

*Corresponding author. yongki@dongguk.ac.kr

Published online in J-STAGE

doi: 10.1254/jphs.08197FP

subjected to MCAO (10, 11). Also, iNOS may contribute to ischemic injury and DNA damage through the formation of peroxynitrite (12). Consistent with a damaging role, hypothermia is associated with reduced microglial generation of both NO and iNOS (13). It has been demonstrated in previous reports that cerebral ischemia up-regulates the inducible forms of NOS and COX-2 in neurons, glial cells, and infiltrating leukocytes in injured brains (14–16).

COX, a rate-limiting enzyme in the metabolism of arachidonic acid into prostanoids, produces prostaglandin H₂, which in subsequent steps, gives rise to prostaglandins with various physiologic functions (17–19) and its toxic effects through prostaglandin E₂ (PGE₂) production (20). Therefore, the inhibition of COX-2 activity during or after the onset of an ischemic stroke and genetic deletion of COX-2 reduce infarct volume (21). Interestingly, iNOS-derived NO production positively regulates COX-2 activity in focal cerebral ischemia (22). These inducible molecules are reversed by nuclear factor-kappaB (NF- κ B) transcriptional activation and increase 24 h after MCAO. The persistent activation of NF- κ B has a deleterious effect after ischemia by mediating neuronal damage. Although the role of NF- κ B activation in relation to cell death or survival is unclear, the blocking of NF- κ B activation can prevent neuronal degeneration caused by stroke-induced ischemia (23).

Recently, there has been increased interest in herbal medicines and their safety and efficacy in many inflammatory diseases. The roots of *Panax notoginseng* (Burk.) F.H. CHEN (Araliaceae [PN]), called notoginseng or sanchi, are widely used to stop hemorrhage in the blood circulation in traditional Korean medicine. Recent studies have shown that PN extracts and major compounds, such as the ginsenosides (Rb1, Rg1, Re1, and Rh1) and the notoginsenoside (R1) exert various pharmacologic activities such as limitation of liver fibrosis (24), hepatic microvascular dysfunction (25), trauma and bleeding due to internal and external injury, immunomodulation (26–28), anti-cancer activities (29, 30), and improvement in myocardial ischemia (31).

Therefore, the present study was conducted to elucidate the neuroprotective effects of the methanol extract of PN in ischemic brain injury using a MCAO rat model. We also determined whether the PN extract inhibits the induction of iNOS and COX-2 in vivo and LPS-stimulated microglia.

Materials and Methods

Preparation and treatment of the plant extract

The roots of *Panax notoginseng* (PN; Yunnan Province, China) were purchased from the Kwangmyung-

dang Herb Co., Ltd., Ulsan, Korea; and they were identified by the herbal medicine botanist Prof. Y.K. Park, Department of Herbology, College of Oriental Medicine (Ducom), Dongguk University. The voucher specimens of PN (PN-M0709) have been deposited in the Herbarium of Ducom. Dried PN (500 g) was extracted using an 80% methanol aqueous mix (3L). The methanol extract filtrate was evaporated *in vacuo*, freeze-dried (105 g, 21.02% of yield), and stored at -20°C until used.

PN was dissolved in $1 \times$ phosphate-buffered saline (PBS) and administered intraperitoneally (i.p.) at a dose of 50 mg/kg 2 h after the onset of MCAO. Rats in the control group received vehicle in the same volume and with the same time schedule as PN-treated animals.

Animals

Experiments were carried out using 8–9-week-old male Sprague-Dawley rats (280–320 g) purchased from KOATECH (Pyungtaeck, Kyungki, Korea). All animal procedures were approved by the animal welfare guidelines issued by the Korean National Institute of Health and the Korean Academy of Medical Sciences. Rats were housed under controlled conditions ($22 \pm 3^{\circ}\text{C}$, lighting with humidity of $60 \pm 5\%$) and allowed free access to food and water.

Transient focal brain ischemia

The procedure for transient MCAO was as previously described (32). Briefly, rats were anesthetized with 5% isoflurane in oxygen/nitrogen ($\text{N}_2\text{O}/\text{O}_2$ [20/80]). During surgical procedures, a mixture of 1.5% isoflurane and $\text{N}_2\text{O}/\text{O}_2$ (20/80) was used to maintain anesthesia under controlled conditions ($37 \pm 5^{\circ}\text{C}$) using a rectal thermometer and a heating pad (FHC, Inc., Bowdoinham, ME, USA). The common right carotid artery (CCA), external carotid artery (ECA), and internal carotid artery (ICA) were exposed through a midline neck incision, and a filament with a rounded tip and a nylon suture (0.3 mm in diameter) was introduced into the CCA and ICA to block the origin of the MCA. After 90 min of occlusion, the nylon suture was withdrawn to allow reperfusion.

Measurement of infarct volume

At 24 h following the onset of ischemia, animals were anesthetized by 5% isoflurane inhalation and sacrificed by decapitation. Brains were removed quickly and chilled in ice-cold saline for 5 min, and coronal tissue sections (2×7 mm) were obtained using a tissue slicer. The slices were immersed in a saline solution containing 2% 2,3,5-triphenyltetrazolium chloride (TTC; Sigma, St. Louis, MO, USA) for 10 min at 37°C and then

preserved in 4% paraformaldehyde solution. Brain slices were photographed using a digital camera and quantified using an image analyzing program (Image J 1.38X; NIH, Bethesda, MD, USA). The infarct area in each slice was calculated by subtracting the normal ipsilateral areas from the contralateral hemisphere to reduce errors due to cerebral edema and was presented as the percentage of the infarct to the area of the contralateral hemisphere.

Immunohistochemistry

At 24 h following the onset of ischemia, animals were anesthetized and brains were perfusion-fixed with 4% paraformaldehyde after transcardial perfusion with saline. The fixed brains were embedded with paraffin and then sectioned (5 μ m). Paraffin-embedded sections were placed in boiled citrate buffer (pH 6.0) in a microwave oven (650 – 720 W). For detection of microglia, sections were pre-incubated in 3% H₂O₂ and then incubated for 2 h at room temperature with horseradish peroxidase (HRP)-conjugated *Griffonia simplicifolia* isolectin-B4 (GSA I-B₄, 5 μ g/ml; Sigma). After washing in 1 $\times$ PBS, the binding site of isolectin was visualized using the diaminobenzidine (DAB) system and counterstained with hematoxylin (Vector Laboratories, Burlingame, CA, USA). Finally, sections were dehydrated in graded ethanol, immersed in xylene, and cover-slipped. The GSA I-B₄-positive cells were counted in the penumbra of the cortex from three independent experiments and presented as the number of immunopositive cells/0.15 mm².

In double-labeling experiments, sections were blocked in normal 5% donkey serum for 1 h at room temperature and then incubated with anti-iNOS and -COX-2 antibodies (1:100; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Sections were revealed using Cy3-conjugated donkey anti-rabbit IgG (1:250; Chemicon International, Inc., Temecula, CA, USA) and isothiocyanate (FITC)-labeled CD11b (1:100; Chemicon International, Inc.). For detection of NF- κ B, sections were incubated with anti-NF- κ B p65 (1:300; Santa Cruz Biotechnology, Inc.) for overnight at 4°C and then with Cy3-conjugated donkey anti-rabbit IgG (1:250; Chemicon International, Inc.) for 1 h at room temperature. Sections were also counter-stained with 4'-6-diamidino-2-phenylindole (DAPI, Vector Laboratories). All sections were analyzed using a Zeiss Olympus microscope and the images were captured using a Kontrol Digital Camera (Mel Soel Microscopes, Ltd., Hicksville, NY, USA). At least 10 different fields were recorded for each measurement and a representative image was presented in the figures.

Cell cultures

Primary mixed glial cells were prepared from 1 – 3-

day-old postnatal Sprague-Dawley rat pups, as described previously (33). Briefly, the brain cortices were pre-treated in 0.25% trypsin solution containing of 1 mM EDTA and 1% papain for 5 min in a 37°C incubator, passed through a nylon mesh (100 μ m), and plated at 2×10^5 cells/cm² in 0.01% poly-D-lysine-coated T-75 flasks. The cultures were fed every 3 – 4 days with DMEM containing 10% FBS, 2 mM glutamine, and antibiotics. On days 6 – 7, the cultures were shaken on a rotary shaker at 200 rpm for 30 min. The suspended cells were seeded in a 100-mm culture dish and maintained in medium containing 10% serum. Based on GSA I-B₄-positive immunostaining, microglial cells were determined to be >95% pure.

BV-2 mouse microglial cells were donated by Dr. H.S. Han of Kyungpook National University (Daegu, Korea). The cells were maintained in DMEM containing 10% FBS, 2 mM glutamine, and penicillin/streptomycin (100 units/ml; HyClone, Logan, UT, USA) at 37°C in 5% CO₂ – 95% O₂ in a humidified incubator. Cytotoxic effects of treatments were determined by measuring the metabolic activity of cells with a 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay.

Measurement of nitric oxide (NO) levels

The nitrite concentration in culture supernatants was measured by the Griess reaction as an indicator of NO production. Briefly, primary microglial cells or BV-2 cells (2×10^5 cells/ml in a 6-well plate) were pretreated with the PN extract for 30 min and then stimulated with LPS (1 μ g/ml, 0111:B4; Sigma) for 18 h. Culture supernatant (0.1 ml) was mixed with an equal volume of Griess reagent [1% sulfanilamide and 0.1% *N*-(1-naphthyl)-ethylenediamine dihydrochloride in 5% phosphoric acid] and incubated for 15 min at room temperature. The nitrite concentration was determined by measuring absorbance at 540 nm using a Microplate reader (ASYS Hitech GmbH, Eugendorf, Austria), and the concentration was determined using a standard curve prepared with sodium nitrite.

Measurement of PGE₂ levels

PGE₂ levels in the culture supernatant were quantified using a commercially available enzyme immunosorbent assay (EIA) kit (Cayman Chemical Co., Ann Arbor, MI, USA) by following the instructions provided by the manufacturer. The PGE₂ levels were determined based on the amount of PGE₂ detected by the EIA kit using a standard curve.

Reverse transcription (RT)-PCR

Total RNA from the cells was isolated using Trizol Reagent (Invitrogen, Carlsbad, CA, USA). cDNA was

synthesized according to the manufacturer's instructions for the reverse transcription kit (Promega Co., Madison, WI, USA) and amplified with a multiplex PCR kit (Promega). Conditions for amplification were as follows: initial denaturation for 2 min at 95°C, 30 cycles of 95°C for 30 s, 53°C–64°C for 30 s, and 72°C for 10 min. RT-PCR products were analyzed by 1% agarose gel electrophoresis, stained with ethidium bromide, and scanned with Gel-Doc (Bio-Rad, Richmond, CA, USA). The specific primers used in this study were as follows: iNOS (accession no. 010927) forward primer, 5'-AAT GGC AAC ATC AGG TCG GCC ATC ACT-3'; iNOS reverse primer, 5'-GCT GTG TGT CAC AGA AGT CTC G AA C TC-3'; COX-2 (accession no. 011198) forward primer, 5'-GGA GAG ACT ATC AAG ATA GT-3'; COX-2 reverse primer, 5'-ATG GTC AG TAG ACT TTT ACA-3'; GAPDH forward primer, 5'-CTC GTG GAG TCT ACT GGT GT-3'; and GAPDH reverse primer, 5'-GTC ATC ATA CTT GGC AGG TT-3' (GAPDH). iNOS and COX-2 mRNA levels were normalized vs. GAPDH mRNA in the same samples.

Immunoblot analysis

After incubation in the presence and absence of stimuli, the brain tissues or cultured cells were used for immunoblot analysis. Brain tissues were homogenized in lysis buffer (50 mM Tris-HCl [pH 7.6]) containing 50 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 10% glycerol, and protease inhibitor cocktail (Roche Diagnostics, Basel, Switzerland). The homogenates were centrifuged at $11,500 \times g$ for 10 min to precipitate the protein and boiled for 5 min in loading buffer. The cells were harvested and lysed in an ice-cold lysis buffer. The protein concentration was determined using a commercially available protein assay dye (Bradford reagent) from Bio-Rad (Hercules, CA, USA). Proteins (30 μ g/lane) were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Mersham Biosciences, Piscataway, NJ, USA). Blots were blocked for 1 h in 5% non-fat dry skim milk in TBS-T buffer (10 mM Tris-HCl containing 150 mM NaCl and 0.1% Tween-20), washed, and then incubated with anti-iNOS (1:1000, Santa Cruz Biotechnology, Inc.) and anti-COX-2 (1:1000, Santa Cruz Biotechnology, Inc.) in 5% BSA TBS-T buffer overnight at 4°C and then washed. The blots were incubated with a secondary horseradish peroxidase-conjugated antibody (1:5000, Santa Cruz Biotechnology, Inc.) for 1 h at room temperature. The blots were developed using an enhanced chemiluminescence detection kit (Pierce Biotechnology, Inc., Rockford, IL, USA), according to the manufacturer's instructions, with subsequent exposure of the membrane to X-ray film. Densitometric analysis

was performed using an image analyzing program (Image J 1.38X).

Statistical analysis

Statistical analysis was performed using Graph-pad Prism 3.0 (GraphPad Software, La Jolla, CA, USA), unless stated otherwise. Data are expressed as the mean $\pm$ S.E.M. of three independent experiments. Comparisons among means of groups were analyzed with a two-tailed Student's *t*-test of unpaired variables. *P*-values <0.05 were considered to be statistically significant.

Results

PN extract attenuates infarct volume after MCAO

The neuroprotective effects of the PN extract were evaluated by measuring infarction volumes 24 h after reperfusion. PN extract (50 mg/kg body weight) was administered just after the onset of ischemia in this study. The selected dose had no significant effects on body weight between the control and PN groups (data not shown).

Figure 1A shows typical photographs of TTC-stained sections from vehicle- and PN extract-treated animals, showing that the treatment reduced infarction. As seen in Fig. 1B, the infarct volumes, which were based on all 7 slices from 6 different animals, decreased significantly from $41.7 \pm 5.37\%$ (vehicle) to $23.82 \pm 8.9\%$ (PN).

PN extract inhibits microglial activation after MCAO

To determine the effects of the PN extract on the inflammatory process in the post-ischemic brain, we studied activated microglia in the cortex penumbra of the ischemic brain using GSA I-B₄ (a microglia marker) immunostaining.

In the vehicle group, GSA I-B₄-positive microglia were enriched in the cortex penumbra of the ischemic hemisphere (Fig. 2A) and progressed further, revealing the morphology of the phagocytic microglia along with the state of activation (Fig. 2C). In contrast, GSA I-B₄-positive cells were nearly undetectable in the brains of PN extract-treated rats, in which but a few ramified microglia and resting microglia were detected (Fig. 2D). Moreover, GSA I-B₄-positive cells were decreased significantly from $200 \pm 27.62/\text{mm}^2$ (vehicle) to $86.6 \pm 11.42/\text{mm}^2$ (PN).

PN extract inhibits the expression of iNOS and COX-2 in microglia after MCAO

To further investigate the effects of PN extract on the inflammatory process in the post-ischemic brain, we investigated the expression of inflammatory molecules, such as iNOS and COX-2, in microglia using immuno-

Fig. 1. PN extract administered 2 h after onset of MCAO at a dose of 50 mg/kg reduces brain damage significantly. Brain sections from rats subjected to 90-min occlusion and 24 h reperfusion. PN extract significantly attenuated the ischemic damage at 24 h of reperfusion. The sections were stained with 2% TTC (2,3,5-triphenyltetrazoliumchloride) (A). Effect of PN extract-treated group (n = 6) on infarct volumes are expressed as the percentage of hemisphere volumes and are presented as the means $\pm$ S.E.M. ** $P < 0.05$, compared to the vehicle-treated group (n = 6) (B).

Fig. 2. PN extract suppress the activation of microglia in 24 h after one set of MCAO. Activation of microglia in the vehicle- and PN extract-treated groups after MCAO was visualized by GSA I-B₄ immunostaining. Activated microglia was detected in the cerebral cortex of the ischemic hemisphere (A), which subsequently became transformed to phagocytic microglia (C). Activated microglia was barely detectable when PN extract was administered at 2 h after onset of MCAO (D). The numbers of GSA I-B₄-positive cells in the indicated areas (0.43 $\times$ 0.35 mm) were obtained by scoring the GSA I-B₄-positive cells in a blind manner from three groups of independent experiments and presented as values of the mean $\pm$ S.E.M. ** $P < 0.01$, compared to the vehicle-treated group (B). Bar = 50 μ m.

histochemistry with CD11b, the histochemical marker of activated microglia. The expressions of iNOS and COX-2 were detected in the cortex penumbra of the ischemic hemisphere 24 h after MCAO.

CD11b- and iNOS-positive cells were increased in ischemic brains of the vehicle-treated control rats, but reduced in PN extract-treated rats (Fig. 3: A–H). The presence of expression of iNOS in the ischemic cortex

was supported by immunoblot analysis, as shown in Fig. 3I. In addition, the PN extract substantially reduced the number of CD11b- and COX-2-positive cells in the ischemic cortex compared with vehicle-treated control rats (Fig. 4: A–H). The PN extract also inhibited the protein levels of COX-2 in the ischemic brains (Fig. 4I). As a result, the PN extract significantly reduced the expression of iNOS compared with the vehicle-treated

Fig. 3. Comparison of vehicle-treated group (A – C) with PN extract-treated group (E – G) shows immunoreactivity for CD11b antigen (A, E) and iNOS (B, F) in the cerebral cortex of the ischemic hemisphere and the same section areas shown by identical patterns of nuclei (DAPI) (D, H). Co-localization of iNOS (red) and CD11b (green) in the PN extract-treated group (G) showed very faint structures as compared with the vehicle-treated group (C). The small scale box represents each arrow. PN inhibits the expression of iNOS protein as determined by immunoblot analysis 24 h after onset of MCAO. Expression of iNOS was quantified by densitometry analysis and expressed in bar charts. Values are each the mean $\pm$ S.E.M. of three different experiment groups ($n = 3$). [#] $P < 0.05$, compared to the sham-treated group and ^{*} $P < 0.05$, compared to the vehicle-treated group (I). Bar = 50 μ m.

Fig. 4. Comparison of vehicle-treated group (A – D) with PN extract-treated group (E – H) shows immunoreactivity for CD11b antigen (A, E) and COX-2 (B, F) in the cerebral cortex of the ischemic hemisphere and the same section areas shown by identical patterns of nuclei (DAPI) (D, H). Co-localization of COX-2 (red) and CD11b (green) in the PN extract-treated group (G) is much less than the vehicle-treated group (C). The small scale box represents each arrow. PN suppressed COX-2 expression of protein levels by Western blotting 24 h after one set of MCAO. Expression of COX-2 was quantified by densitometry analysis and expressed in bar charts. Values are each the mean $\pm$ S.E.M. of three different experiment groups.

Fig. 5. Nuclear and cytoplasmic expression of the NF- κ B p65. Immunohistochemical staining of the NF- κ B p65 in the brain sections of rats that underwent MCAO. The NF- κ B p65 staining is present in the nucleus and cytoplasm after MCAO. The vehicle-treated group showed nuclear translocation (A – C) and treatment with the PN extract preserved this (D – F) at 24-h reperfusion after 90-min MCAO. NF- κ B p65 was detected in the nucleus (a) and cytosol (b); the percentage of p65 in the nucleus in the indicated areas ($0.15 \times 0.22 \text{ mm}^2$) is representative of similar results obtained from three groups of animals and presented as the means $\pm$ S.E.M. $^{\#}P < 0.001$, compared to the vehicle-treated group (G). Bar = $20 \mu\text{m}$.

group in the cortex, but COX-2 was not significantly reduced by immunoblot analysis.

PN inhibits NF- κ B p65 translocation from the cytosol to the nucleus after MCAO

NF- κ B is an important molecule in signaling pathways that controls the synthesis and release of inflammatory mediators, such as iNOS and COX-2, in the inflammatory response of ischemic brains. Therefore, we investigated the effects of the PN extract on the nuclear localization of NF- κ B expression by immunohistochemistry.

In the vehicle-treated group, the expression of the NF- κ B p65 subunit increased in the nucleus, but was reduced in PN extract-treated rats (Fig. 5: A – F). As seen in Fig. 5G, the number of NF- κ B p65-positive cells in the nucleus significantly decreased from $55.96\% \pm 25.63\%$ (vehicle) to $23.82 \pm 8.9\%$ in nucleus of ischemic cortex (PN; $n = 3$, $P < 0.001$).

PN extract inhibits NO and PGE₂ production in LPS-stimulated microglia

The inhibition of inflammatory mediators, such as NO and PGE₂, from microglia/macrophages is believed to protect neurons from cell death. Therefore, we

evaluated the potential effects of the PN extract in the regulation of NO and PGE₂ production from activated microglia in vitro.

Unstimulated primary microglia and BV-2 cells secrete a basal level of NO and PGE₂, but the secretion of NO and PGE₂ increased after exposure to LPS ($1 \mu\text{g/ml}$) for 18 h (Fig. 6: A and B, respectively). The PN extract significantly inhibited the production of both NO and PGE₂ in a concentration-dependent manner. Importantly, no cytotoxicity was observed at any of the concentrations of the PN extract tested, as assessed by the 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide assay (data not shown). Also, no significant differences were observed in cell recoveries between PN extract- and control-treated groups in both cell types.

PN extract inhibits iNOS and COX-2 expression in LPS-stimulated microglia

To further investigate the anti-inflammatory mechanism of the PN extract in the activated microglia, we determined the expression of inducible inflammatory enzymes, such as iNOS and COX-2, by RT-PCR and immunoblot analysis.

As shown in Figs. 7 and 8, untreated cells and the PN

Fig. 6. Effects of PN on the production of NO and PGE₂ in LPS-stimulated BV-2 and primary microglia. Cells were stimulated with LPS (1 μg/ml) 30 min after PN (10–100 μg/ml) pretreatment and incubated for 18 h. The amount of NO was determined using the Griess reagent and a standard curve created using NaNO₂ in culture medium (A). The levels of PGE₂ in the media were detected using an enzyme immunoassay (B). Each independent experiment is indicated by the mean ± S.E.M. **P*<0.05 and ***P*<0.01, compared with LPS alone.

extract alone expressed detectable levels of iNOS mRNA (Fig. 7A) and protein expression (Fig. 8A) or were undetected, while their expression was increased after LPS stimulation. However, PN extract significantly attenuated the LPS-induced expression of iNOS mRNA and protein in both cell types (*P*<0.05).

Moreover, the mRNA (Fig. 7B) and protein expression (Fig. 8B) of COX-2 was reduced in both LPS-stimulated cells. These results indicate that the PN extract inhibited NO and PGE₂ production via regulation at the transcriptional level of the iNOS and COX-2 genes in activated microglia, respectively.

PN extract inhibits NF-κB activation in LPS-stimulated microglia

We further investigated whether PN extract acts to block the activation of the NF-κB pathway, which is implicated in the transcriptional regulation of inflammatory mediators in activated microglia, by immunoblot analysis.

As shown in Fig. 9, LPS stimulation induced trans-

location of the NF-κB p65 subunit into the nucleus; however, the effect of LPS was significantly inhibited by the treatment of the PN extract in a concentration-dependent manner. This data implies the potential role of the NF-κB pathway as the possible mechanism by which the PN extract suppresses inflammatory mediators such as NO and PGE₂.

Discussion

In the present study, the administration of the PN extract showed neuroprotective potential and anti-inflammatory effects on brain ischemic injuries induced by MCAO through the inhibition of inflammation-related events, including microglial activation and the expression of inflammatory inducible enzymes such as iNOS and COX-2. Rats subjected to cerebral ischemia–reperfusion showed typical hallmarks of neuronal death and cerebral inflammation with the up-regulation of microglial activation and the induction of iNOS and COX-2 in vulnerable regions of the CNS (34, 35). However, unlike neurons, glial cells survive transient ischemia and subsequently give rise to prominent gliosis in regions of neuronal death.

Microglia constitutes approximately 5%–10% of the total glial cell population, and unlike other glial cells in the brain, their activation is observed in regions of neuronal death after the onset of cerebral ischemia (36–38). In the present study, rats administered the PN extract (50 mg/kg, i.p.) 2-h post-occlusion showed a 23.82 ± 8.9% reduction in infarction volume compared with vehicle-treated control rats. In addition, GSA I-B₄-positive microglia was up-regulated 24 h after ischemia in the cortex penumbra of the ischemic hemisphere, whereas the PN extract decreased the number of GSA I-B₄-positive cells. Furthermore, accumulation of amoeboid microglia/macrophages was inhibited by PN extract-treatment.

NO is a free radical produced from L-arginine by nitrite oxide synthases (NOSs), and it is an important cellular second messenger. Especially, iNOS-derived NO release is one of the major contributing factors during the inflammatory process in cerebral ischemic injury (9, 10) and provides a means of assessing the effects of drugs on basic inflammation. Therefore, pharmacologically-selective inhibitors of NO/iNOS can attenuate infarct volume in ischemic brain (39, 40). In this study, iNOS mRNA and protein levels were found to be up-regulated in ischemic hemispheres, whereas PN extract administration inhibited iNOS induction in the ischemic rat brain. In addition, experiments with primary microglia and BV-2 microglial cells showed that the PN extract reduced NO production, indicating that

Fig. 7. Effects of PN on the mRNA expression of iNOS and COX-2 in LPS-stimulated BV-2 and primary microglia. The cells were treated with PN (20, 50, and 100 µg/ml) in the presence or absence of LPS (1 µg/ml) for 6 h. Total RNA was isolated, and mRNA levels of iNOS (A) and COX-2 (B) were measured by RT-PCR. The ratio of mean densitometry values of iNOS and COX-2 were quantified by densitometry analysis and expressed in bar charts. GAPDH expression was used as an internal control. Values are each the mean $\pm$ S.E.M. of three different experiment groups, * P <0.05, compared with LPS alone.

they inhibit proinflammatory microglial activation.

COX-2, among representative genes modulated early after onset of stroke, has become the focus of attention because it is the rate-limiting enzyme involved in arachidonic acid metabolism, thereby generating prostaglandins and thromboxanes that play important roles in the inflammatory response (41). In rodents, as well as humans, cerebral ischemia up-regulates COX-2 expression in neurons, blood vessels, and inflammatory cells in the injured brain (42, 43). Therefore, the administration of a selective COX-2 inhibitor attenuated the elevation of PGE₂ and reduced the infarct in a model of MCAO (15). COX-2 products may contribute to NMDA-induced neuronal injury and the pathogenesis of NO after ischemia (44, 45). Thus, iNOS-derived NO production has been shown to contribute to COX-2 activity in

the ischemic brain (46). Therefore, the inhibition of iNOS could serve as a neuroprotectant through COX-2 inhibition just before the start of delayed neuronal death (47). Following cerebral ischemia and reperfusion, COX-2 induction is closely associated with post-ischemic inflammation and NO production. In the present study, COX-2 was up-regulated in the ischemic brain, which is consistent with the findings of previous studies (48, 49); and the administration of the PN extract inhibited this induction. Furthermore, PN significantly reduced the production of COX-2-derived PGE₂ through the regulation of gene transcription levels in primary microglia and BV-2 cells. This result suggests that the neuroprotective effects of the PN extract may be due to the inhibition of COX-2 induction in cerebral ischemia.

Fig. 8. Effects of PN on the induction of iNOS and COX-2 protein in LPS-stimulated BV2 and primary microglia. The cells were treated with PN (20, 50, and 100 $\mu\text{g/ml}$) in the presence or absence of LPS (1 $\mu\text{g/ml}$) for 18 h. Cell lysates were extracted, and protein levels of iNOS (A) and COX-2 (B) were analyzed by immunoblot analysis. The ratio of mean densitometry values of iNOS and COX-2 were quantified by densitometry analysis and expressed in bar charts. Actin expression was used as an internal control. Values are each the mean $\pm$ S.E.M. of three different experiment groups, * $P < 0.05$, compared with LPS alone.

NF- κ B is a key regulator of inflammation and the immune response in brain ischemia and its activation has been shown to be instrumental in the up-regulation of iNOS and COX-2 or other inflammatory molecules that are damaging in stroke (50). In this study, the administration of the PN extract inhibited the translocation of NF- κ B p65 into the nucleus in the ischemic brain. In addition, experiments with BV-2 microglial cells showed that the PN extract reduced NF- κ B p65 translocation to the nucleus, indicating inhibition of proinflammatory microglial activation. Transient focal ischemia caused a substantial up-regulation of NF- κ B p65 after post-ischemic injury due to induction in reactive glial cells in the damaged areas (51, 52). Round reactive microglia/macrophages strongly immunoreactive to the NF- κ B p65 were abundant within the cortex penumbra of the ischemic hemisphere.

Previous studies have reported that PN contains more

than 30 different types of saponins including ginsenoside (Rb1, Re, Rg1, and Rh1) and notoginsenoside R1 (53). Previous studies have proved that *Panax notoginseng* saponin inhibits platelet aggregation in the patients with blood hyperviscosity syndrome (4) and improves I/R-induced hepatic microcirculation disturbance and vascular endothelium function (54), and notoginsenoside R1 inhibits leukocyte rolling and adhesion by inhibiting the expression of adhesion molecules in neutrophils (24). However, which compounds from PN extract corresponding to neuroprotective property in ischemic brain had not been elucidated; it is significant that PN extract reduces the infarction in ischemic brain and inhibits inflammation by activated microglia. Furthermore, the molecular mechanisms responsible for the neuroprotection of the extract had been addressed in the study. Further research is obviously required to identify the main active compounds in the extract, as well as to

Fig. 9. Effects of PN on the translocation of NF-κB p65 subunit into the nuclei of LPS-stimulated BV-2 cells. The cells were treated with PN (20, 50, and 100 μg/ml) in the presence or absence of LPS (1 μg/ml) for 15 min. The nuclear proteins from the cell analysis were used for the detection of p65 by immunoblot analysis. The ratio of mean densitometry values of p65 in the nucleus was quantified by densitometry analysis and expressed in bar charts. Actin expression was used as an internal control. Each value indicated the mean ± S.E.M. and is representative of results obtained from three different experiment groups, * $P < 0.05$, ** $P < 0.01$ compared with LPS alone.

determine its neuroprotective properties against ischemic stroke.

In conclusion, we showed that the PN extract exhibited significant neuroprotective effects in rats with transient focal ischemia, and that the neuroprotective effect of the PN extract appears to involve the inhibition of the induction of iNOS and COX-2 up-regulation by activation of NF-κB in the ischemic brain. Our findings suggest that the roots of *P. notoginseng* have a neuroprotective effect in the ischemic brain, which is due to the inhibition of inflammation and of microglial activation, and can be used as potential therapeutic agent against inflammation in ischemic stroke.

Acknowledgment

This study was supported by a grant of the Oriental Medicine R&D Project, Ministry of Health & Welfare, Rep. of Korea (B080027 to Y.K. Park).

References

- Klijn CJ, Hankey GJ. Management of acute ischaemic stroke: new guidelines from the American Stroke Association and European Stroke Initiative. *Lancet Neurol.* 2003;2:698–701.
- Thomas WE. Brain macrophages: evaluation of microglia and their functions. *Brain Res.* 1992;17:61–74.
- El Khoury J, Hickman SE, Thomas CA, Loike JD, Silverstein SC. Microglia, scavenger receptors, and the pathogenesis of Alzheimer's disease. *Neurobiol Aging.* 1998;19:S81–S84.
- Wang Q, Tang XN, Yenari MA. The inflammatory response in

- stroke. *J Neuroimmunol.* 2006;184:53–68.
- Stoll G, Jander S, Schroeter M. Inflammation and glial responses in ischemic brain lesions. *Prog Neurobiol.* 1998;2:149–171.
- Kim EJ, Kwon KJ, Park JY, Lee SH, Moon CH, Baik EJ. Effects of peroxisome proliferator-activated receptor agonists on LPS-induced neuronal death in mixed cortical neurons: associated with iNOS and COX-2. *Brain Res.* 2002;1–2:1–10.
- Zhang Z, Chopp M, Powers C. Temporal profile of microglial response following transient (2h) middle cerebral artery occlusion. *Brain Res.* 1997;744:189–198.
- Yu YM, Kim JB, Lee KW, Kim SY, Han PL, Lee JK. Inhibition of the cerebral ischemic injury by ethyl pyruvate with a wide therapeutic window. *Stroke.* 2005;36:2238–2243.
- Samdani AF, Dawson TM, Dawson VL. Nitric oxide synthase in models of focal ischemia. *Stroke.* 1997;28:1283–1288.
- Iadecola C. Bright and dark sides of nitric oxide in ischemic brain injury. *Trends Neurosci.* 1997;20:132–139.
- Goyagi T, Goto S, Bhardwaj A, Dawson VL, Hurn PD, Kirsch JR. Neuroprotective effect of σ1-receptor ligand 4-phenyl-1-(4-phenylbutyl) piperidine (PPBP) is linked to reduced neuronal nitric oxide production. *Stroke.* 2001;32:1613–1620.
- Cui J, Holmes EH, Greene TG, Liu PK. Oxidative DNA damage precedes DNA fragmentation after experimental stroke in rat brain. *FASEB J.* 2000;14:955–967.
- Han HS, Qiao Y, Karabiyikoglu M, Giffard RG, Yenari MA. Influence of mild hypothermia on inducible nitric oxide synthase expression and reactive nitrogen production in experimental stroke and inflammation. *J Neurosci.* 2002;22:3921–3928.
- Miettinen S, Fusco FR, Yrjänheikki J, Keinänen R, Hirvonen T, Roivainen R, et al. Spreading depression and focal brain ischemia induce cyclooxygenase-2 in cortical neurons through N-methyl-D-aspartic acid-receptors and phospholipase A2. *Proc Natl Acad Sci USA.* 1997;94:6500–6505.
- Nogawa S, Zhang F, Ross ME, Iadecola C. Cyclo-oxygenase-2 gene expression in neurons contributes to ischemic brain damage. *J Neurosci.* 1997;17:2746–2755.
- Iadecola C, Forster C, Nogawa S, Clark HB, Ross ME. Cyclooxygenase-2 immunoreactivity in the human brain following cerebral ischemia. *Acta Neuropathol.* 1999;98:9–14.
- Hayaishi O. Molecular mechanisms of sleep-wake regulation: roles of prostaglandins D2 and E2. *FASEB J.* 1991;5:2575–2581.
- Kaufmann WE, Andreasson KI, Isakson PC, Worley PF. Cyclooxygenases and the central nervous system. *Prostaglandins.* 1997;54:601–624.
- Matsuoka Y, Okazaki M, Zhao H, Asai S, Ishikawa K, Kitamura Y. Phosphorylation of c-Jun and its localization with heme oxygenase-1 and cyclooxygenase-2 in CA1 pyramidal neurons after transient forebrain ischemia. *J Cereb Blood Flow Metabol.* 1999;19:1247–1255.
- Manabe Y, Anrather J, Kawano T, Niwa K, Zhou P, Ross ME, et al. Prostanoid, not reactive oxygen species, mediate COX-2-dependent neurotoxicity. *Ann Neurol.* 2004;55:668–675.
- Nakayama M, Uchimura K, Zhu RL, Nagayama T, Rose ME, Stetler RA, et al. Cyclooxygenase-2 inhibition prevents delayed death of CA1 hippocampal neurons following global ischemia. *Proc Natl Acad Sci USA.* 1998;95:10954–10959.
- Nogawa S, Forster C, Zhang F, Nagayama M, Ross ME, Iadecola C. Interaction between inducible nitric oxide synthase and cyclooxygenase-2 after cerebral ischemia. *Proc Natl Acad*

- Sci USA. 1998;95:10966–10971.
- 23 Mattson MP, Goodman Y, Luo H, Fu W, Furukawa K. Activation of NF- κ B protects hippocampal neurons against oxidative stress-induced apoptosis: evidence for induction of manganese superoxide dismutase and suppression of peroxynitrite production and protein tyrosine nitration. *J Neurosci Res.* 1997;49:681–697.
 - 24 Wei Y, Fan JM, Pan LP. Effect of *Panax notoginseng* saponins on human kidney fibroblast. *Zhongguo Zhong Xi Yi Jie He Za Zhi.* 2002;22:47–49.
 - 25 Park KM, Kim YS, Jeong TC, Joe CO, Shin HJ, Lee YH, et al. Nitric oxide is involved in the immunomodulating activities of acidic polysaccharide from *Panax ginseng*. *Planta Med.* 2001;67:122–126.
 - 26 Gao H, Wang F, Lien EJ, Trousdale MD. Immunostimulating polysaccharides from *Panax notoginseng*. *Pharm Res.* 1996;13:1196–1200.
 - 27 Rhule A, Navarro S, Smith JR, Shepherd DM. *Panax notoginseng* attenuates LPS-induced pro-inflammatory mediators in RAW264.7 cells. *J Ethnopharmacol.* 2006;106:121–128.
 - 28 Jin UH, Park SG, Suh SJ, Kim JK, Kim DS, Moon SK, et al. Inhibitory effect of *Panax notoginseng* on nitric oxide synthase, cyclo-oxygenase-2 and neutrophil functions. *Phytother Res.* 2007;21:142–148.
 - 29 Yang ZG, Sun HX, Ye YP. Ginsenoside Rd from *Panax notoginseng* is cytotoxic towards HeLa cancer cells and induces apoptosis. *Chem Biodivers.* 2006;3:187–197.
 - 30 Wang CZ, Xie JT, Zhang B, Ni M, Fishbein A, Aung HH, et al. Chemopreventive effects of *Panax notoginseng* and its major constituents on SW480 human colorectal cancer cells. *Int J Oncol.* 2007;31:1149–1156.
 - 31 Lei XL, Chiou GC. Cardiovascular pharmacology of *Panax notoginseng* (Burk) F.H. Chen and *Salvia miltiorrhiza*. *Am J Chin Med.* 1986;14:145–152.
 - 32 Longa EZ, Weinstein PR, Carlson S, Cummins R. Reversible middle cerebral artery occlusion without craniectomy in rats. *Stroke.* 1989;20:84–91.
 - 33 Frei A, Cottier C, Wunderlich P, Lüdin E. Glycerol and dextran combined in the therapy of acute stroke; a placebo-controlled, double-blind trial with a planned interim analysis. *Stroke.* 1987;18:373–379.
 - 34 Jander S, Schroeter M, Stoll G. Role of NMDA receptor signaling in the regulation of inflammatory gene expression after focal brain ischemia. *J Neuroimmunol.* 2000;109:181–187.
 - 35 Liu SJ, Zhou SW, Xue CS. Effect of tetrandrine on neutrophilic recruitment response to brain ischemia/reperfusion. *Acta Pharmacol Sin.* 2001;22:971–975.
 - 36 Gehrmann J, Bonnekoh P, Miyazawa T, Hossmann KA, Kreutzberg GW. Immunocytochemical study of an early microglial activation in ischemia. *J Cereb Blood Flow Metab.* 1992;12:257–269.
 - 37 Le W, Rowe D, Xie W, Ortiz I, He Y, Appel SH. Microglial activation and dopaminergic cell injury: an in vitro model relevant to Parkinson's disease. *J Neurosci.* 2001;21:8447–8455.
 - 38 Ryu JK, Shin WH, Kim J, Joe EH, Lee YB, Cho KG, et al. Trisialoganglioside GT1b induces in vivo degeneration of nigral dopaminergic neurons: role of microglia. *Glia.* 2002;38:15–23.
 - 39 Jiang MH, Kaku T, Hada J, Hayashi Y. 7-Nitroindazole reduces nitric oxide concentration in rat hippocampus after transient forebrain ischemia. *Eur J Pharmacol.* 1999;380:117–121.
 - 40 Nanri K, Montécot C, Springhetti V, Seylaz J, Pinard E. The selective inhibitor of neuronal nitric oxide synthase, 7-nitroindazole, reduces the delayed neuronal damage due to forebrain ischemia in rats. *Stroke.* 1998;29:1248–1253.
 - 41 Vane JR, Bakhle YS, Botting RM. Cyclooxygenases 1 and 2. *Annu Rev Pharmacol Toxicol.* 1998;38:97–120.
 - 42 Ohtsuki T, Kitagawa K, Yamagata K, Mandai K, Mabuchi T, Matsushita K, et al. Induction of cyclooxygenase-2 mRNA in gerbil hippocampal neurons after transient forebrain ischemia. *Brain Res.* 1996;736:353–356.
 - 43 Candelario-Jalil E, González-Falcón A, García-Cabrera M, Alvarez D, Al-Dalain S, Martínez G, et al. Assessment of the relative contribution of COX-1 and COX-2 isoforms to ischemia-induced oxidative damage and neurodegeneration following transient global cerebral ischemia. *J Neurochem.* 2003;86:545–555.
 - 44 Kittaka M, Giannotta SL, Zelman V, Correale JD, DeGiorgio CM, Weiss MH, et al. Attenuation of brain injury and reduction of neuron-specific enolase by nicardipine in systemic circulation following focal ischemia and reperfusion in a rat model. *J Neurosurg.* 1997;87:731–737.
 - 45 Hewett SJ, Uliasz TF, Vidwans AS, Hewett JA. Cyclooxygenase-2 contributes to N-methyl-D-aspartate-mediated neuronal cell death in primary cortical cell culture. *J Pharmacol Exp Ther.* 2000;293:417–425.
 - 46 Surh YJ, Chun KS, Cha HH, Han SS, Keum YS, Park KK, et al. Molecular mechanisms underlying chemopreventive activities of anti-inflammatory phytochemicals: down-regulation of COX-2 and iNOS through suppression of NF- κ B activation. *Mutat Res.* 2001;480–481:243–268.
 - 47 Endoh M, Maiese K, Wagner J. Expression of the inducible form of nitric oxide synthase by reactive astrocytes after transient global ischemia. *Brain Res.* 1994;651:92–100.
 - 48 Yokota C, Kaji T, Kuge Y, Inoue H, Tamaki N, Minematsu K. Temporal and topographic profiles of cyclooxygenase-2 expression during 24 h of focal brain ischemia in rats. *Neurosci Lett.* 2004;357:219–222.
 - 49 Zhao Y, Patzer A, Herdegen T, Gohlke P, Culman J. Activation of cerebral peroxisome proliferator-activated receptors gamma promotes neuroprotection by attenuation of neuronal cyclooxygenase-2 overexpression after focal cerebral ischemia in rats. *FASEB J.* 2006;20:1162–1175.
 - 50 Khan M, Sekhon B, Giri S, Jatana M, Gilg AG, Ayasolla K, et al. S-Nitrosoglutathione reduces inflammation and protects brain against focal cerebral ischemia in a rat model of experimental stroke. *J Cereb Blood Flow Metabol.* 2005;25:177–192.
 - 51 Gabriel C, Justicia C, Camins A, Planas AM. Activation of nuclear factor-kappaB in the rat brain after transient focal ischemia. *Brain Res Mol Brain Res.* 1999;65:61–69.
 - 52 Herrmann O, Baumann B, de Lorenzi R, Muhammad S, Zhang W, Kleesiek J, et al. IKK mediates ischemia-induced neuronal death. *Nat Med.* 2005;11:1322–1329.
 - 53 Du Q, Jerz G, Waibel R, Winterhalter P. Isolation of dammarane saponins from *Panax notoginseng* by high-speed counter-current chromatography. *J Chromatogr A.* 2003;1008:173–180.
 - 54 Park WH, Lee SK, Kim CH. A Korean herbal medicine, *Panax notoginseng*, prevents liver fibrosis and hepatic microvascular dysfunction in rats. *Life Sci.* 2005;76:1675–1690.