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**Effects of Fimasartan, One of Angiotensin
Receptor Blockers, on Tissue Damages and
Inflammation After Focal Ischemia in Rat Brain**

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ABSTRACT

Effects of Fimasartan, One of Angiotensin Receptor Blockers, on Tissue Damages and Inflammation After Focal Ischemia in Rat Brain

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Background and Purpose: Fimasartan is a newly developed angiotensin receptor blocker (ARB), and evidences suggests that fimasartan may have protective effects during myocardial infarction or atherosclerosis. However, the application of regular-dose ARBs during ischemic stroke should be used cautiously (due to lessons learned from other ARBs). In this context, we investigated the effects of low-dose fimasartan on tissue damage and inflammation after focal ischemia in rat brain.

Materials and Methods: To evaluate anti-inflammatory effects of fimasartan, oxygen-glucose deprivation (OGD) was applied in cultured astrocytes and fimasartan was treated. In *in vivo* experiments,

noninvasive blood pressure monitoring was performed in Sprague-Dawley rats which fimasartan or phosphate-buffered saline (PBS) were administered for 4 weeks. A low-dose (0.5 mg/kg) and regular doses (1 or 3 mg/kg) of fimasartan was administered intraorally for 4 weeks, and we induced ischemia-reperfusion injury in rats. Infarct volume and the number of TUNEL-positive apoptotic cells were measured at 7 days. Functional outcomes were evaluated by modified limb placing test. To identify the anti-inflammatory effect of fimasartan, the number of inflammatory cells (OX6-positive cells) was measured. To evaluate inflammatory makers, I κ B and cyclooxygenase-2 (COX-2) were measured via Western blot analyses at 48 hours. Especially for low-dose fimasartan (0.5 mg/kg), the effects of post-treated fimasartan on infarct volume was also evaluated.

Results: During OGD, fimasartan reduced the inflammatory markers in astrocytes: nuclear translocation of NF- κ B, degradation of I κ B, and expression of COX-2. After the administration of low-dose (0.5 mg/kg) fimasartan, blood pressure did not decrease compared to the phosphate-buffered saline (PBS)-control with MCA occlusion (MCAO) group. The infarct volume and ischemic cell death were reduced in the low-dose fimasartan-treated group (46 ± 41 for 0.5 mg/kg and 153 ± 47 mm³ for PBS-control with MCAO; $P < 0.01$) but not in the regular-dose groups. Low-dose fimasartan treatment improved functional recovery after ischemia and significantly decreased mortality. In our study, fimasartan reduced the degradation of I κ B (whose degradation is

a marker for NF- κ B activation) and the formation of an inflammatory end-product, COX-2. As a result, the recruitment of inflammatory cells in the peri-infarct area decreased after low-dose, long-term treatment. After induction of transient MCAO, low dose fimasartan (0.5 mg/kg) was administered for 3 days per oral route. The infarct volume at 3 days after MCAO decreased significantly in low-dose post-treated group compare to PBS-control with MCAO (110.8 ± 46.1 versus 162.7 ± 42.3 mm³; $P < 0.05$).

Conclusion: We have demonstrated that low-dose, long-term fimasartan pre-treatment improved outcomes after focal ischemia in the brain via a reduction of inflammation. These low-dose fimasartan may have beneficial effects on tissue damage in post-treatment after MCAO.

Keywords: Fimasartan, Stroke, Focal ischemia, Inflammation, Angiotensin receptor blocker

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INTRODUCTION

Fimasartan (BR-A-657) is the ninth developed angiotensin receptor blocker (ARB) from Korea [1]. Fimasartan is a series of pyrimidin-4(3H)-one derivatives as losartan analogues, and fimasartan has about six hundred-fold affinity for the angiotensin II receptor type 1 (AT1) compared to equivalent doses of losartan [2, 3]. In addition to greater potency, fimasartan is rapidly absorbed following oral administration, and the time to peak plasma concentration ranging 0.5-3 hours with comparable safety profile [1]. Thus, it has been used as an anti-hypertensive drug in Korea since 2011 after approval by the Korean Food and Drug Administration. In addition to lowering blood pressure (BP), there are a few experimental studies suggesting fimasartan has protective effects during cardiovascular diseases. Fimasartan decreased infarct volume and improved functional outcomes during rat myocardial infarction [4], and it prevented the progression of atherosclerosis in injured vessels in a rabbit model [5]. However, the effect of fimasartan on focal ischemia in the brain has not been investigated.

Stroke is one of the leading causes of mortality worldwide including Korea, and presents a disease burden to patients' families and society due to disability after stroke [6]. Despite these detrimental outcomes, stroke treatment has been limited to recanalization therapy, and there is no practical neuroprotective agent [7, 8]. Some ARBs demonstrated neuroprotective effects in animal models against ischemic stroke [9-11].

However, one of those ARBs (candesartan) did not demonstrate any beneficial effect for ischemic stroke patients when administered in the acute phase and may have even been harmful [12]. It has been postulated that this failure may originate from lowered BP in the candesartan treatment group because tissue hypo-perfusion might accelerate ischemic damage in the acute phase after stroke. In some diseases, such as diabetic nephropathy in which the relative hypotension may promote the progression of the disease, the effects of low-dose ARBs have been studied [13, 14]. Furthermore, physicians usually choose antihypertensive drugs according to comorbidities of patients and adverse events of drugs, not by the outcomes of cardiovascular events related to hypertension. However, a recent retrospective cohort study showed that pre-stroke administration of ARBs might improve the outcomes of stroke [15]. In this context, we designed the study to support experimental background of beneficial effects of a pre-treated novel ARB, fimasartan, and wanted to give a help for physicians to choose one of various antihypertensive drugs to improve the outcomes of ischemic stroke, because some patients with high risk of stroke may have the event of ischemic stroke even though they took anti-hypertensive drugs regularly.

Angiotensin II receptor blockers (ARBs), are mostly used to treat hypertension, and exert pleiotropic effects to protect against cerebral injury by ameliorating brain inflammation [16-18]. Previous studies have illustrated the neuroprotective effects of the ARBs by reducing inflammatory processes for the treatment of stroke [19-21]. The

administration of low doses of candesartan to normotensive rats decreased the release of proinflammatory cytokines, the hormone aldosterone, and LPS-induced mRNA expression, which exhibits broad anti-inflammatory effects [22]. Telmisartan, which can function as a partial agonist of peroxisome proliferator-activated receptor gamma (PPAR- γ) greatly reduced inflammation and exerted protective effects in stroke experiments [23, 24]. Furthermore, in *in vitro* system, fimasartan decreased inflammation via suppression of NF- κ B signal pathways [25]. Especially in hemolysate-treated astrocytes, this anti-inflammatory effects were related to ERK and Akt signal pathways, which was verified by the inhibition of each signal pathway [26]. In this context, we investigated the effects of fimasartan, a novel ARB, on tissue damages and inflammation after focal ischemia in rat brain.

MATERIALS AND METHODS

Animals and Experimental Groups

Male Sprague-Dawley rats (Koatech, Seoul, Republic of Korea) weighing between 200 and 220 g, were used in these experiments. All animal studies were performed according to the National Institutes of Health Guide for the Institutional Animal Care and Use Committee of the Biomedical Research Institute at Seoul National University Hospital. We administered fimasartan at a low-dose (0.5 mg/kg) and a regular dose (1 or 3 mg/kg) or phosphate-buffered saline (PBS) orally for 4 weeks once per day before the induction of ischemia and reperfusion in the middle cerebral artery (MCA). Fimasartan is used as a hypertensive drug for humans between 30 mg and 120 mg. Considering higher ratio of body surface area per body weights in rats than humans, 0.5 mg/kg was correlated with approximately 15-20 mg for humans, which was decided as a maximal dose for pleiotropic effects of fimasartan without BP lowering. After induction of transient MCA occlusion (MCAO), we ceased the fimasartan administration to prevent BP reductions after ischemia (Figure 1). In the post-treatment study, low dose fimasartan (0.5 mg/kg) were administered orally three times after induction of MCAO: immediately after MCAO, post 1 day, and post 2 day. Infarct volumes were measured at 7 days after MCAO in pre-treatment studies, and 3 days after MCAO in post-treatment experiments.

Focal Ischemia-reperfusion Model

Focal cerebral ischemia-reperfusion was induced with a minor modification of the endovascular internal carotid artery (ICA) suture method [27, 28]. After inhalation of 3% isoflurane in 30% oxygen and 70% air, the left common carotid artery (CCA) was exposed at its bifurcation using a midline cervical incision. The external carotid artery (ECA), ICA, and CCA were ligated using a 5-0 silk suture. The CCA was then transected, and a 5-0 nylon monofilament suture (with its tip rounded by heating) was inserted into the CCA. To occlude the origins of the MCA and proximal anterior cerebral artery, the suture was advanced into the ICA for a distance of 20 mm. The suture was secured in place using a ligature, and the wound was closed. The monofilament was removed 60 min after the occlusion. The animals were allowed food and water ad libitum. Rectal temperature was maintained at $37 \pm 0.5^{\circ}\text{C}$ using a thermistor-controlled heating blanket. Sham operations were performed to make a negative control group (n = 4 for Nissl and n = 2 for TTC staining).

Measurement of Infarct Volumes

After cardiac perfusion-fixation with 4% paraformaldehyde in 0.1 mol/L PBS, the brains were removed quickly and cut into 30- μm -thick coronal sections on a freezing microtome. Ten brain sections were mounted onto glass slides, and processed for Nissl staining for measurement of

infarct volumes, and to demonstrate infarct areas clearly, TTC (2,3,5-triphenyltetrazolium chloride) staining was performed (n = 3 for each group). The infarct volumes were measured using an image analysis program, ImageJ (National Institutes of Health, Bethesda, MD).

Astrocyte Cell Culture and Oxygen-Glucose Deprivation

Mouse brain astrocytes (Astrocytes Type I clone; ATCC, CRL-2541) were maintained in Dulbecco's modified Eagle's medium (WELGENE Inc., Daegu, Republic of Korea). The medium was changed once daily. Cells were incubated in a humidified incubator maintained at 37°C with an atmosphere of 5% CO₂. Cells were culture in 80-cm² flasks overnight to 80-90% confluence. For oxygen-glucose deprivation (OGD), astrocytes were incubated in glucose-free Locke's buffer in an oxygen-free chamber for 6 and 24 hours respectively. According to the previous studies measuring toxicity and anti-inflammatory effects in hemolysate-treated astrocytes, 30 ng/mL fimasartan was treated in astrocytes in fimasartan-treated group during OGD.

In situ Labeling of DNA Fragmentation

Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) was performed with the use of a commercially available kit as described previously [29]. Sections were incubated in a TdT-labeling reaction mixture for 90 min, colored with DAB solution,

and counterstained with methyl green. A single axial section through the center of the ischemic lesion was analyzed. Eight sampling regions were placed along the periphery. TUNEL-positive cells were identified and counted. Total counts in these sampling regions were converted into cell densities for quantification and comparison between the treatment and control groups.

Behavioral Testing and Mortality Check

This test was a modified version of a test described by a previous study [30]. The limb-placing test was used to evaluate the outcome of recovery on postoperative days 1, 3, 7, and 14. The test assesses the sensorimotor integration of the forelimb and the hind limb by checking responses to tactile and proprioceptive stimulation. In the first task, the rat was suspended 10 cm over a table, and the forelimb stretch towards the table was observed. In the second test, the rat was positioned towards the table and its forelimbs were placed on the table. Next, the rats were placed along the table edge to check for lateral placement of the forelimb (third task). In the fourth task, the rat was again positioned towards the table with the hind limbs just over the table edge. Each hind limb was pulled down and gently stimulated by pushing towards the side of the table. The 4 tasks were scored in the following manner: normal performance, 0 points; incomplete performance, 1 point; no performance, 2 points. A total of 8 points indicated maximal neurological deficit, and 0 points indicated normal

performance. The modified limb placing tests were also performed 1 hour after induction of MCAO to evaluate the adequateness of modeling for focal ischemia. We excluded rats with 0 point at that time point for the further analysis because of inadequate modeling for the MCAO. The mortality was checked 28 days after induction of transient MCAO.

Measurement of Blood Pressures

The BP was recorded using a CODA Non-invasive Blood Pressure System (Kent Scientific Corporation, Torrington, CT). The BP is recorded by a band attached to the tail (homologated by Bland-Altman testing) [31]. This method is recommended by the American Heart Association as a measuring guide for laboratory animals [32]. Non-invasive BP monitoring was performed on days -28, -27, -25, -21, -14, -7, just before MCAO induction. After MCAO, non-invasive BPs were measured on days 1, 3, and 7. Invasive BPs were obtained via the femoral artery once before ischemia (after fimasartan administration for 28 days).

Immunofluorescent Staining and Cell Quantification

Immunofluorescent staining of brain tissue was performed using cryopreserved 40- μ m coronal sections. Each section was incubated with 0.5% bovine serum albumin/0.3% Triton-X followed by 10% normal

serum in PBS for 1 hour for blocking. Sections with a primary antibody were placed at 4°C for 16 hours. After washing, each section was subsequently incubated for 2 hours at room temperature with the fluorophore-conjugated secondary antibody. Monoclonal antibodies against MHC class II Ia (Ox6; Santa Cruz Biotech, Santa Cruz, CA) labeled activated microglia/macrophages. Stained cells were then examined under a confocal laser scanning biological microscope (LSM 410 META; Carl Zeiss, Jena, Germany).

Quantitative analysis of the positively stained cells was performed in the peri-infarct regions by two independent investigators (H-KP and DK) who were masked to the group allocations. To count activated microglia/macrophages, 16 high-power fields were taken from the sections through the center of the infarct lesion 7 days after transient MCAO. Total counts in the measured sections were converted into cell densities for comparison between fimasartan treatment and control groups according to our established protocol [33].

Western Blot Analysis

The rats were killed via decapitation, and the brains were immediately extracted 48 hours after the induction of transient MCAO. After the centrifugation of hemisphere homogenates, 50 µg of protein was separated on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel and transferred to nitrocellulose membranes. These membranes were incubated in blocking buffer (5% skim milk in 50

mmol/L Tris PH 7.5, 0.15 mmol/L NaCl, 0.05% Tween-20) and the blots were probed with antibodies recognizing NFκB p65 (Abcam, Cambridge, UK) IκB (Cell Signaling Tech., Danvers, MA), and cyclooxygenase-2 (COX-2; BD Biosciences, Franklin Lakes, NJ). Immunoreactivity was visualized by enhanced chemiluminescence, and the relative optical densities were compared to the mean values of the control group.

Nuclear Protein Extraction

For the analysis of NFκB translocation, astrocyte nuclear protein extracts were prepared using hypotonic lysis buffer A (10 mM HEPEPS, PH7.6, 10 mM KCl, 1 mM DTT, 0.1 mM EDTA, and 0.5 mM phenylmethylsulfonyl fluoride) for 10 min on ice and vortexed for 10 s. The homogenate was spun by centrifugation at 12,000 g for 10 min. The pellet containing nuclear fraction was resuspended in buffer C (20 mM HEPEPS, PH7.6, 1 mM EDTA, 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 25% glycerol, and 0.4 M NaCl) for 30 min on ice. The supernatant containing nuclear proteins were collected by centrifugation by 13,000 G for 20 min and stored at -80°C. To assay the nuclear protein translocation of NFκB, equal amounts (40 μg) of protein from the nuclear fractions were analyzed by western blot using an anti-NFκB p65 antibody (1:500). The protocol for western blot analysis is described above.

Statistical Analysis

The values are presented as the means \pm standard deviations. The data were analyzed with the nonparametric Mann-Whitney U test for unpaired samples between two groups, and the non-parametric Kruskal-Wallis H test was used for multiple groups. To compare each group after the Kruskal-Wallis H test, the Bonferroni correction was performed as post hoc test. A two-tailed value of $P < 0.05$ was considered significant. The survival analysis was performed according to the log-rank test. All statistical analyses were performed using SPSS 21.0 (SPSS Inc. Chicago, IL).

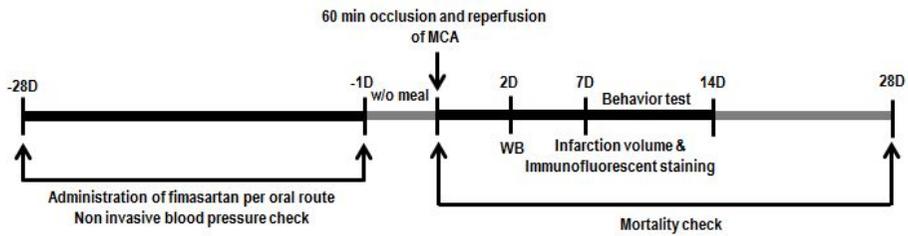


Figure 1. Schematic diagram of the pre-treated *in vivo* study protocols.

For Sprague-Dawley rats, low-dose (0.5 mg/kg) or regular doses (1 or 3 mg/kg) were administered for 4 weeks via an oral route before the induction of transient middle cerebral artery (MCA) occlusion. At 2 days, western blot (WB) analyses were performed. At 7 days immunofluorescent staining was performed, and infarct volume was measured. Behavior tests were performed for 14 days after induction of transient MCA occlusion, and mortality was censored at 28 days. Blood pressure was monitored during the pre-treatment and follow-up period.

RESULTS

Inflammatory Markers in Oxygen-Glucose Deprived Astrocytes

During OGD, the expression of NF- κ B was constant. However, compared to 6 hr OGD, nuclear translocation of NF- κ B increased at 24 hr after OGD. Fimasartan reduced the nuclear translocation of NF- κ B significantly at both 6 hr and 24 hr after OGD ($P < 0.05$; Figure 2). The degradation of I κ B (another marker of activation of NF- κ B) decreased in fimasartan-treated astrocytes compared to OGD-only cells ($P < 0.05$; Figure 3). Although the expression of COX-2 rose time-dependently after OGD, fimasartan suppressed the expression of COX-2 significantly in both 6 hr and 24 hr OGD ($P < 0.05$; Figure 4).

Blood Pressure: Pre-treatment and Follow-up Period

The mean BPs decreased in the regular-dose fimasartan groups at 3 days, but the mean BPs in the low-dose fimasartan were not different from PBS-controls with MCAO via non-invasive monitoring (Figure 5A). Because of a one-day diet restriction prior to focal ischemia, the mean BPs in all groups were lower compared to the resting mean BPs in pre-treatment period. With the single-time invasive monitoring just before focal ischemia, all BPs including the systolic, diastolic and

mean, decreased in the regular-dose fimasartan group, but did not decrease in the low-dose fimasartan group compared with PBS-controls with MCAO (Figure 5B, C, D). After inducing focal ischemia, the mean BPs increased in all groups. The mean BPs in the regular-dose fimasartan groups returned to the level of the low-dose and control groups 3 days after inducing focal ischemia because we ceased fimasartan administration after ischemia to minimize the possible harmful effects of low BP.

Infarct Volume and Ischemic Cell Death

Sham operations did not make any infarct lesions in the brain (Figure 6). The infarct volume decreased in the low-dose pre-treatment groups compared to the PBS-control with MCAO group (46 ± 41 for 0.5 mg/kg and 153 ± 47 mm³ for control; *P* by Bonferroni correction between two groups < 0.01 ; Figure 6). However, the infarct volumes in the regular-dose groups were not different from the PBS-control with MCAO group (95 ± 58 for 1 mg/kg and 103 ± 42 mm³ for 3 mg/kg; *P* by Bonferroni correction = 0.21 for 1 mg/kg and 0.12 for 3 mg/kg compared to the control group). Based on this result, we evaluated ischemic cell death, functional outcomes, mortality, and inflammatory changes between two groups (low-dose and control) to minimize the number of animals sacrificed. Ischemic cell deaths also decreased in the low-dose fimasartan group compared to the PBS-control with MCAO group as determined by TUNEL staining (578 ± 109 for 0.5 mg/kg and

1270 ± 156 cells/mm² for control group, $P < 0.05$; Figure 7).

Functional Outcomes and Mortality

Low-dose fimasartan improved functional recovery after focal ischemia compared to the PBS-control with MCAO group. There was a trend for improved functional scores in the low-dose fimasartan group, and the functional recovery was prominent at 14 days after ischemia (2 ± 2 points for 0.5 mg/kg and 5 ± 3 points for control, $P < 0.05$; Figure 8A). The mortality rate decreased significantly in the low-dose fimasartan group compared with the PBS-control with MCAO (P by Log-rank test < 0.05 ; Figure 8B).

Inflammatory Cell Recruitment and Inflammatory Markers

Ox6-stained microglia/macrophages were less frequently found in the periphery around the lesion, and the density in the low-dose fimasartan-treated group was lower than the PBS-control with MCAO group (Figure 9A). According to quantitative analysis, the fimasartan-treated group exhibited a lower number of Ox6-positive cells (169 ± 54 versus 647 ± 167 cells/mm²) than the PBS-control with MCAO group (Figure 9B).

After ischemia, I κ B was degraded on the lesion hemisphere compared to the contralateral brain in PBS-control with MCAO animals (relative optical density on the lesion side for I κ B = 0.5 ± 0.1). In the low-dose

fimasartan-treated group, the degradation of I κ B was suppressed to the level of the contralateral brain in controlgroup ($P < 0.05$; Figure 10A, B). The density of COX-2, the inflammatory end-product after ischemia, in the infarcted hemisphere was 1.6-fold higher in the control group than the fimasartan-treated group ($P < 0.05$; Figure 10A, C).

Infarct Volume in Post-treatment of Fimasartan

After induction of transient MCAO, low dose fimasartan (0.5 mg/kg) was administered for 3 days per oral route. The mean BPs did not decrease in low-dose treated group compared with control group (Figure 11A). The infarct volume at 3 days after MCAO decreased significantly in low-dose post-treated group compare to PBS-control with MCAO (110.8 ± 46.1 versus 162.7 ± 42.3 mm; $P = 0.018$; Figure 11B).

NF-κB in oxygen-glucose deprived astrocytes

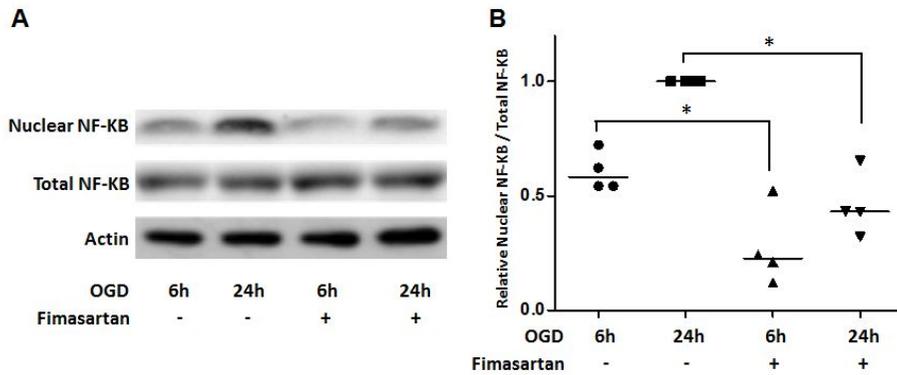


Figure 2. NF-κB in oxygen-glucose deprived astrocytes. The expression P65 NF-κB was constant after oxygen-glucose deprivation (OGD). However, the relative ratio of nuclear NF-κB and total NF-κB (a marker of nuclear translocation of NF-κB) was higher in 24 hr OGD than 6 hr. Fimasartan decreased the relative ratio of nuclear NF-κB and total NF-κB at both 6 hr and 24 hr after OGD ($*P < 0.05$).

IκB in oxygen-glucose deprived astrocytes

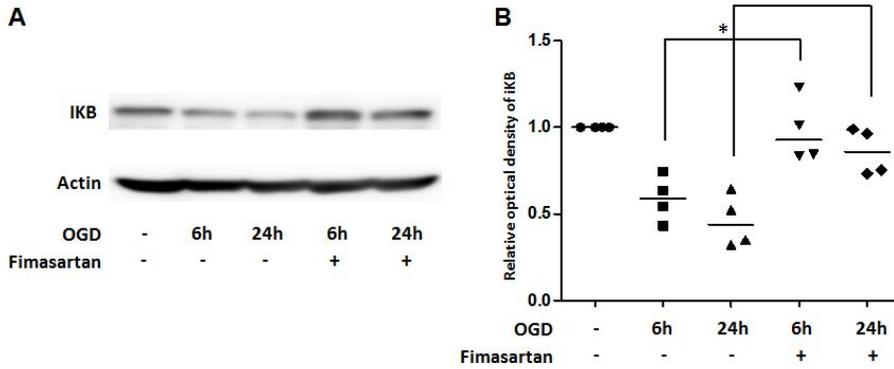


Figure 3. IκB in oxygen-glucose deprived astrocytes. After oxygen-glucose deprivation (OGD), IκB was degraded in time-dependent manner. The degradation of IκB (one of markers for activation of NF-κB) decreased in fimasartan-treated astrocytes compared with OGD-only cells at both 6 hr and 24 hr ($*P < 0.05$).

COX-2 in oxygen-glucose deprived astrocytes

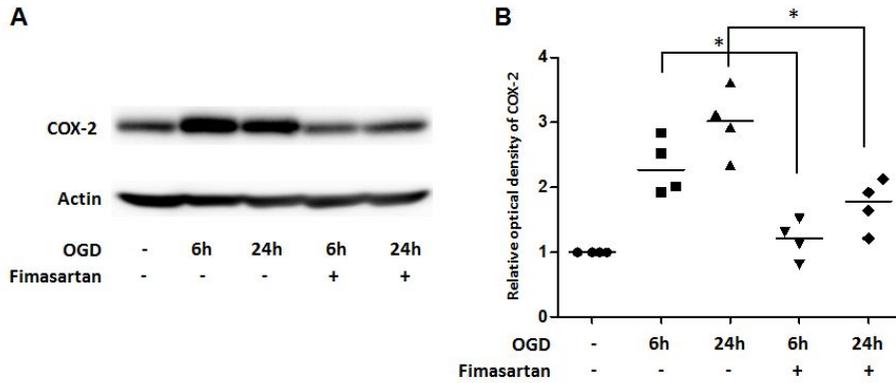


Figure 4. Cyclooxygenase-2 in oxygen-glucose deprived astrocytes.

Cyclooxygenase-2 (COX-2), one of inflammatory end-products increased significantly in oxygen-glucose deprivation (OGD) at both 6 and 24 hr. Fimasartan suppressed the expression of COX-2 significantly ($*P < 0.05$).

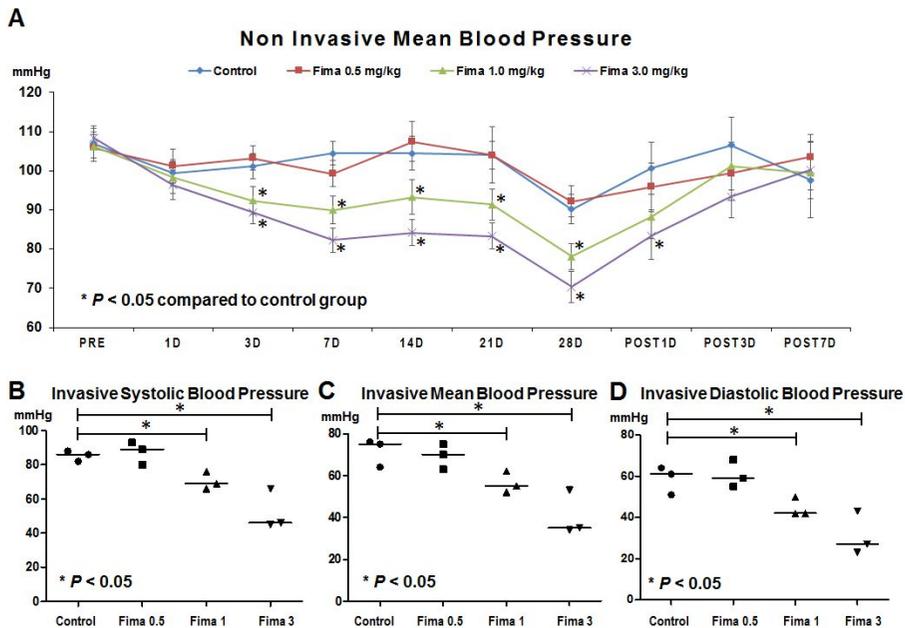


Figure 5. Measurement of Blood pressure. (A) Mean blood pressures (MBPs) were decreased in the regular-dose fimasartan groups at 3 days post-fimasartan administration. The MBPs in the low-dose fimasartan group were not different from the PBS-control with MCAO group ($n = 9$ each). (B) Invasive systolic blood pressures decreased in the regular-dose groups, but did not decrease in the low-dose fimasartan group ($n = 3$ each). (C) Invasive MPBs in the low-dose fimasartan did not decrease compared to PBS-controls with MCAO ($n = 3$ each). (D) Invasive diastolic blood pressures decreased in the regular-dose groups but were not reduced in the low-dose group than PBS-control with MCAO group ($n = 3$ each).

Infarction volume at 7 days after MCAO

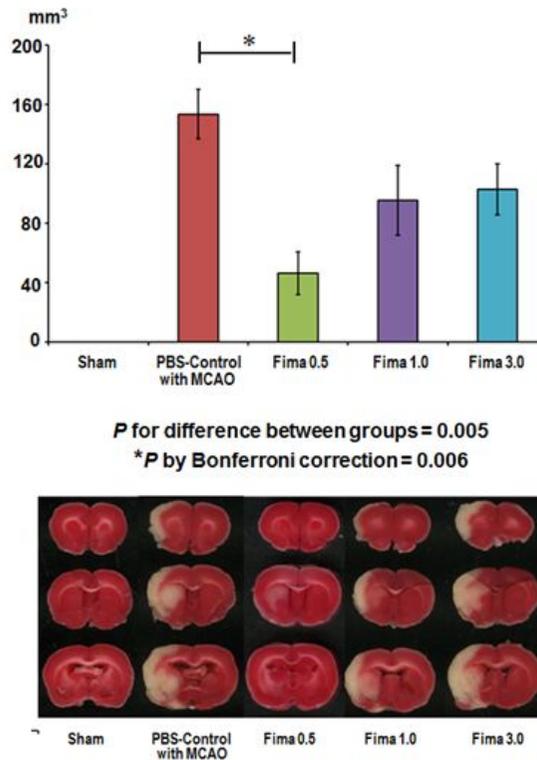
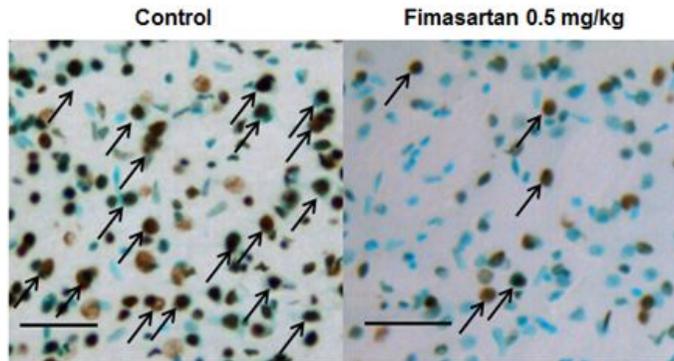


Figure 6. Measurement of infarct volume. Sham operations did not make any infarct lesions in the brain ($n = 6$). The infarct volume in the low-dose fimasartan group (0.5 mg/kg, $n = 8$) decreased significantly compared to the PBS-control with MCAO group ($P < 0.05$ by Bonferroni correction, $n = 8$), but there was no difference between the regular-dose group and PBS-controls with MCAO (P for 1 mg/kg = 0.21 and P for 3 mg/kg = 0.12; $n = 6$ each).

TUNEL Assay



Scale bar=50 μm

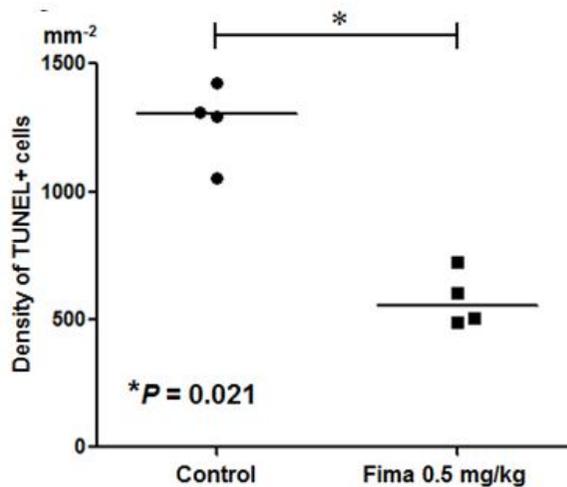


Figure 7. Measurement of ischemic cell death. Ischemic cell death, which evaluated by TUNEL staining, decreased in the low-dose fimasartan group compared with the PBS-control with MCAO group ($P < 0.05$; $n = 4$ each). Representative TUNEL-positive cells (dark brown color) are indicated by black arrows, and TUNEL-negative cells are counter-stained by methyl green (blue color). Scale bar = 50 μm .

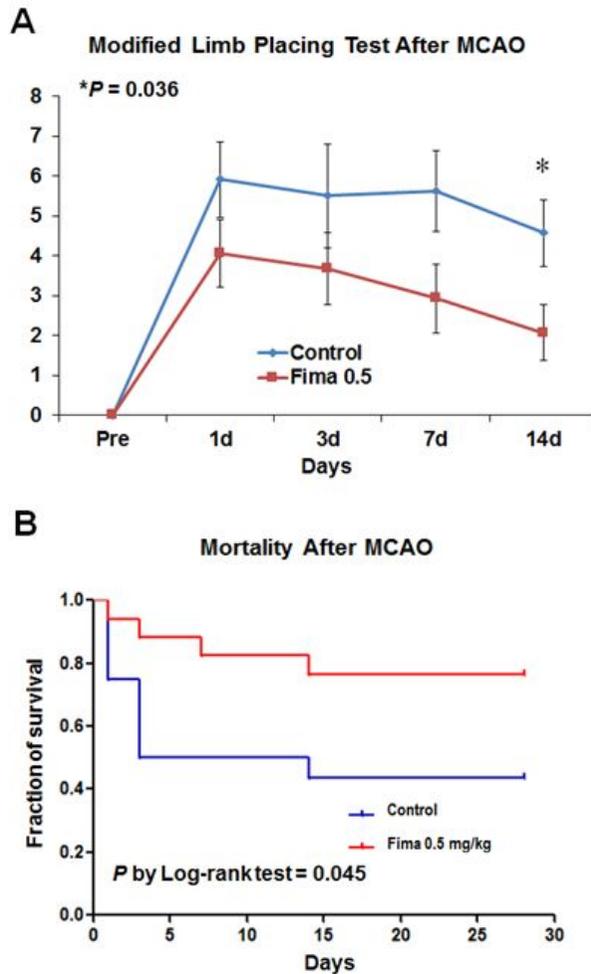


Figure 8. Functional recovery and mortality after focal cerebral ischemia. (A) Low-dose fimasartan improved functional recovery after transient focal ischemia compared to PBS-controls with MCAO. This improvement was prominent at 14 days after ischemia ($P < 0.05$; $n = 17$ for 0.5 mg/kg and $n = 12$ for control). (B) The mortality rate decreased significantly in the low-dose fimasartan group compared with PBS-controls with MCAO (P by Log-rank test < 0.05 ; $n = 18$ each).

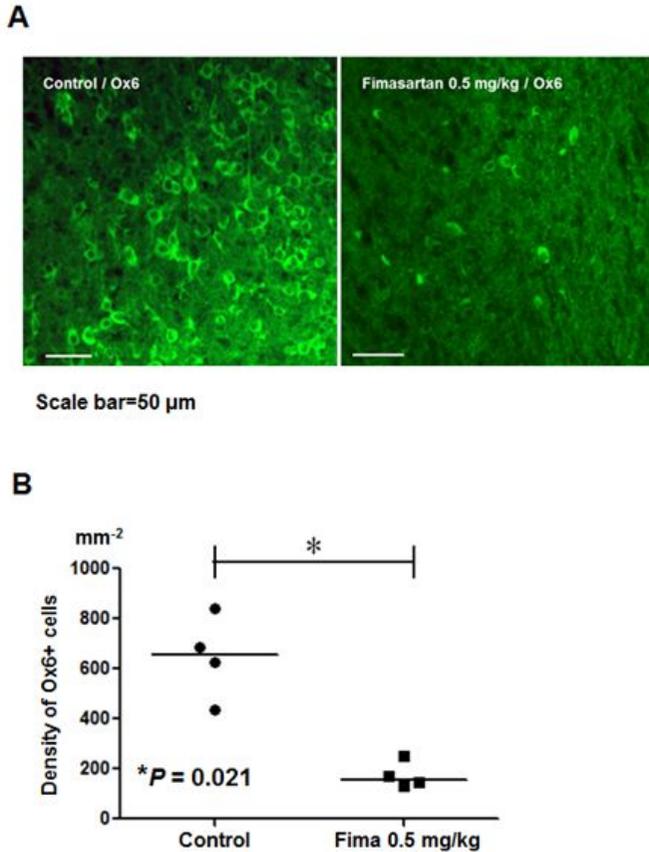


Figure 9. Immunofluorescent staining of inflammatory cells. (A)

Ox6-stained microglia/macrophages (green) were less frequently found in the periphery of the lesion, and the density in the fimasartan-treated group was lower than the PBS-control with MCAO group ($n = 4$ each). Scale bar = 50 μ m. (B) According to the quantitative analysis, the fimasartan-injected group exhibited a lower number of Ox6+ cells ($P < 0.05$) compared to the PBS-control with MCAO group.

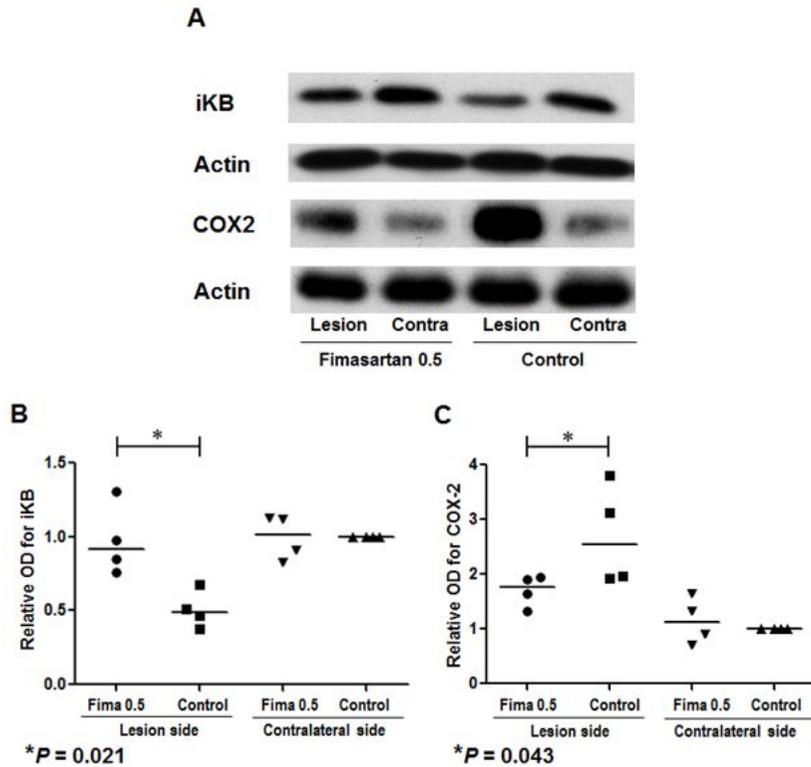
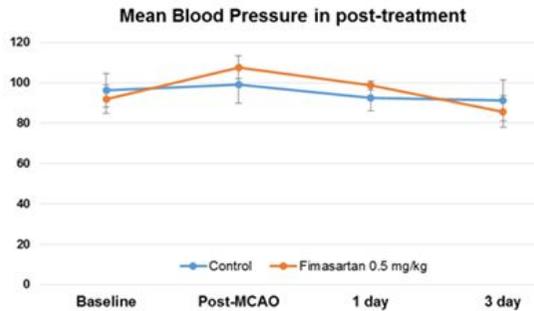


Figure 10. Western blot for inflammatory markers. (A) At 48 hours after ischemia induction, the density of I κ B increased and cyclooxygenase-2 (COX-2) decreased in the fimasartan-treated group compared with the PBS-control with MCAO group (n = 4 each). (B) In the quantitative analyses, the degradation of I κ B was suppressed in the low-dose fimasartan-treated group in the lesion and was comparable to the contralateral brain in the PBS-control with MCAO group compared with lesion side in the PBS-control with MCAO group ($P < 0.05$). (C) The density of COX-2 in the infarcted hemisphere was 1.6-fold higher in the PBS-control with MCAO group compared to the fimasartan-treated group ($P < 0.05$).

A



B

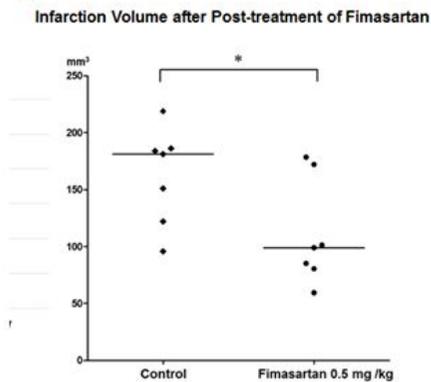


Figure 11. Infarct volumes and blood pressures in post-treatment with low-dose fimasartan. After induction of transient MCAO, low dose fimasartan (0.5 mg/kg) was administered for 3 days per oral route. (A) In noninvasive monitoring for blood pressures, there was not any significant lowering of mean blood pressures in low-dose post-treatment group. (B) The infarct volume at 3 days after MCAO decreased significantly in low-dose post-treatment group compare to PBS-control with MCAO (110.8 ± 46.1 versus 162.7 ± 42.3 mm³; $*P = 0.018$).

DISCUSSION

In this study, Fimasartan decreased the nuclear translocation of NF- κ B, the degradation of I κ B, and the expression of COX-2 in oxygen-glucose deprived astrocytes. Based on these *in vitro* results, we performed *in vivo* tests after induction focal transient ischemia in rat brain. Long-term pre-treated low-dose fimasartan (0.5 mg/kg) decrease infarct volume, and ischemic cell death. Also, it improved the functional outcomes and mortality through reduction of inflammatory reaction after MCAO. Interestingly, regular doses of fimasartan which decreased BPs in normotensive rats could not show these beneficial effects. Furthermore, low-dose fimasartan reduced infarct volume even though it is treated after induction of MCAO.

Fimasartan improved the outcomes after ischemic stroke in the experimental model and decreased inflammation related to ischemic damage. Inflammation is an important process that elicits acute damage after brain ischemia [34]. Compared to other pathologic processes after brain ischemia, inflammation is a potent reaction associated with reactive oxygen species, necrosis, apoptosis, and tissue remodeling. Moreover, it is closely related to various cell types such as microglia/macrophages, endothelial cells, neurons, and glial cells. In a recent *in vitro* study, fimasartan decreased inflammatory reactions in macrophages by reducing a pro-inflammatory transcription factor, NF- κ B, and inflammatory end-products such as iNOS [25]. This effect of

fimasartan may have originated from the blocking of angiotensin II, which has been known to provoke inflammation through AT1 receptor [35]. This kind of anti-inflammatory effect by ARBs was also observed in the brain. Candesartan suppressed the expression of cytokine mRNA in LPS-induced inflammatory brain tissue [36]. In our study, fimasartan reduced the degradation of I κ B, which is a marker for NF- κ B activation, and the formation of an inflammatory end-product, COX-2. As a result, the recruitment of inflammatory cells in the peri-infarct area decreased after long-term fimasartan treatment.

Some other ARBs have shown neuroprotective effects against cerebral ischemia in hypertensive animals [37, 38]. The protective effects focused on the reversal of chronic vascular damage, which was induced by high BPs. In addition to this BP lowering effect, some investigators thought that ARBs might have direct protective effects against focal cerebral ischemia. To evaluate this, a few ARBs were investigated in normotensive animals. Irbesartan which was injected intracerebroventricularly decreased infarct volume, apoptosis and inflammation independent of the systemic effects [9]. According to this observation, candesartan, which was administered via an intravenous or oral route, decreased infarct volume and improved functional outcomes after brain ischemia in normotensive rats [11, 39]. The experiments that delivered candesartan intravenously were mainly focused on post-treatment after ischemia and regular doses of ARBs, which decreased BP. However, in a recent clinical trial, acute treatment with a regular dose of candesartan was not beneficial for ischemic stroke patients

likely due to tissue hypo-perfusion [12]. Similar to this human trial, we observed that low-dose fimasartan was superior to the regular doses. To reveal the protective effect of fimasartan independent of the BP lowering effects, we administered low-dose fimasartan to normotensive animals for a long period before inducing ischemia.

Low-dose ARB treatments have been evaluated in some disease models [40, 41]. Unlike myocardial infarction where high BP is directly harmful to ischemic tissue due to the increased work load of the heart, the brain and kidney are more vulnerable to relative hypotension in ischemic states than the heart. For diabetic nephropathy, the beneficial effects of low-dose ARBs have already been reported [13, 14], and there are ongoing clinical trials investigating the effects of low-dose fimasartan on diabetic nephropathy. Without adequate oxygen and glucose, neurons could not survive for more than a few minutes [42]. In this context, lowering the BP during the acute period after cerebral infarction should be applied cautiously. Low-dose ARB treatment might be considered as a neuroprotective treatment after cerebral infarction, and fimasartan is a good candidate for low-dose treatment because it has highly potent affinity for AT1 compared to other ARBs.

The present experiment had a few limitations. First, the protective effects of fimasartan on focal brain ischemia may not be exclusively caused by the anti-inflammatory effects of fimasartan. According to our observations, reducing inflammation could be one of the important beneficial processes of fimasartan, but other pathologic ways after cerebral ischemia such as apoptosis or disrupted auto-regulation might

be related to the effects of fimasartan. Second, to evaluate the protective effects of fimasartan independent of its BP lowering effects, our experiment was performed in normotensive animals. However, because fimasartan is mainly used as an anti-hypertensive drug, the neuroprotective effects of regular doses of fimasartan after cerebral ischemia in hypertensive animals should be investigated in future experiments.

Taken together, we have demonstrated that low-dose, long-term fimasartan treatment improved outcomes after focal brain ischemia via inflammation reduction. Fimasartan is already being used to treat hypertension in the daily practice of some countries because it has high potency and tolerable safety [1]. From our translational approach with fimasartan, we found a novel and safe therapeutic candidate to protect brain tissue after ischemia. After considering the lessons from experimental and clinical studies with other ARBs [12, 40], we applied an adequate dose of fimasartan so that its pleiotropic effect (independent of BP lowering) could be maximized. We believe that this protective effect of fimasartan will be investigated in human studies for the treatment of ischemic stroke in the near future.

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국문초록

배경: 피마살탄은 국내에서 개발된 새로운 안지오텐신 수용체 차단제로 일부 심근경색 및 동맥경화 동물모델에서 보호효과를 나타내었음. 또한, 일부 *in vitro* 연구에서는 항염증작용이 보고되기도 함. 그럼에도 불구하고 안지오텐신 수용체 차단제를 뇌경색 동물 모델에 적용할 때는 주의를 필요로 하는데, 최근 급성기 뇌경색 환자에서 안지오텐신 수용체 차단제를 적용하였을 때 급격한 혈압저하로 보호효과를 보이는 것에 실패한 임상연구가 보고되기도 함. 이러한 연구결과들을 토대로 본 연구에서는 다른 안지오텐신 수용체 차단제보다 높은 역가를 가지는 피마살탄을 저용량으로 장기간 사용하였을 때 뇌경색 동물 모델에 보호효과를 가지는지를 연구하였으며, 특히 조직 손상과 염증반응에 미치는 영향에 대해 주목하였음.

방법: 피마살탄의 항염증작용을 확인하기 위해 별아교세포에 산소-포도당을 결핍 시킨 이후 발생하는 염증반응의 지표를 확인함. 동물 실험에서는 피마살탄 저농도(0.5 mg/kg)와 상용량(1 mg/kg, 3 mg/kg)을 경구로 백서에 투여하여 4주 동안 비침습적 방법을 통해 혈압을 측정함. 이후 1시간 동안 중대뇌동맥을 폐쇄한 이후 재개통하는 방법으로 백서의 뇌에 국소 뇌허혈을 유도함. 국소 뇌허혈 이후에 뇌경색의 크기, 허혈성 세포사를 7일째 측정하였으며, 이와 더불어, 뇌경색이 유도된 백서의 기능학적 회복과 사망률을 추적 관찰함. 피마살탄의 항염증 작용을 확인하기 위해서, OX-6에 대한 항체를 이용하여 뇌경색 주변으로 염증세포가 동원된 정도를 확인하였

으며, 다른 염증의 마커인 cyclooxygenase-2 (COX-2)와 IκB를 측정하였음. 특히 저농도의 피마살탄의 뇌경색에 대한 보호효과를 확인하기 위해서 저농도 피마살탄(0.5 mg/kg)은 뇌경색 후 투여를 하고 뇌경색의 부피를 측정함.

결과: 별아교세포에 산소와 포도당을 결핍한 이후 피마살탄을 투여한 경우 NF-κB의 세포핵 쪽으로의 전이, IκB의 분해, COX-2의 생성이 피마살탄에 의해 모두 억제됨. 피마살탄이 *in vitro* 시스템에서 염증반응의 지표를 억제함을 확인할 수 있었음. 피마살탄의 저농도(0.5 mg/kg)를 투여하였을 때는 대조군에 비해 혈압의 저하가 없었으며, 피마살탄 저농도를 뇌경색 유도 전 28일간 전처치 한 군에서는 뇌경색의 부피와 허혈성 세포사가 모두 감소함(피마살탄 저농도군: $46 \pm 41 \text{ mm}^3$, 대조군: $153 \pm 47 \text{ mm}^3$; $P < 0.01$). 그러나 상용량의 피마살탄(1 mg/kg, 3 mg/kg)을 투여한 군에서는 상기 보호효과가 관찰되지 않았음. 상기 백서를 추적 관찰하였을 때 저농도의 피마살탄을 투여한 군에서 기능학적 회복이 빨라지며, 생존율이 증가하는 것을 확인함. 또한, 염증반응의 지표를 비교했을 때, 뇌경색이 발생한 대뇌반구에서 피마살탄을 투여했을 때 IκB의 분해가 저하되고, COX-2의 생성이 억제되는 것을 확인함. 이러한 결과를 토대로 저농도의 피마살탄을 뇌경색 유도 후 처리하는 실험을 진행함. 뇌경색 유도 후 저농도(0.5 mg/kg)의 피마살탄을 3일간 처리한 경우 대조군에 비해 뇌경색의 크기가 감소함을 확인함(피마살탄 저농도군: $110.8 \pm 46.1 \text{ mm}^3$, 대조군: $162.7 \pm 42.3 \text{ mm}^3$; $P < 0.05$).

결론: 저농도의 피마살탄을 뇌경색 유도 전 장기간 전처치를 한 경

우 뇌경색의 크기를 감소시키고 예후를 향상시키는 현상을 백서의 국소 뇌허혈 모델에서 확인함. 이러한 보호효과는 항염증작용에 의해 발생함을 별아교세포를 통한 *in vitro* 실험과 뇌조직의 형광염색과 단백질 측정을 통해 확인함. 이러한 저농도의 피마살탄은 전처치뿐만 아니라, 뇌경색 이후에 투여하였을 때도 정상혈압 백서에서 보호효과를 가질 수 있음을 확인함.

주요어: 피마살탄, 뇌졸중, 국소 뇌허혈, 염증, 안지오텐신 수용체 차단제

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