Regulatory function of agmatine in neuroinflammation following cerebral ischemia

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Regulatory function of agmatine in neuroinflammation following cerebral ischemia

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부푼 꿈을 안고 10년 전 해부학에 발을 들여놓은 것이 엊그제 같은데 벌써 10년이란 시간이 흘러 한 과정의 종지부를 찍게 되었습니다. 처음 열정을 가지고 조직학이란 학문에 뛰어들어 한동안 앞만 보고 달리기도 했지만, 초심을 잃고 틈틈이 게으름도 피우고 일탈을 꿈꾸며 보냈던 시간들이 주마등처럼 스쳐갑니다. 그 동안 힘들었던 기억보다는 더 많이 배우지 못하고, 최선을 다하지 못했던 시간과 사람에 대한 아쉬움과 나의 부족함으로 인해 늦어진 학위를 연세대학교가 나를 너무 사랑해서 놔주지 않는다고 농담처럼 이야기 하곤 했는데 이제는 그 사랑을 끊을 때가 된 것 같아 서운한 마음이 앞섭니다.

제가 학위를 받는 다기 보다 오히려 선생님께서 하나 더 학위를 받으셔도 될 만큼 저보다 더 속 끓이시고 맘고생이 심하셨을 이종은 교수님께 깊은 감사를 드립니다.

엘레강스가 흐르지만 강한 카리스마 때문에 항상 긴장의 끈을 놓을 수 없게 하시는 내 인생의 표본이신 박경아 선생님, 그리고 냉철한 외모 때문에 다가서기 힘들지만 알고 보면 마음이 따뜻한 이원택 선생님께도 긴 시간동안 부족했던 부분 하나하나를 짚어주시고 가르쳐 주신 은혜에 감사드립니다.

사업가의 머리와 과학자의 가슴을 지닌 김재환 선생님, 스스로에게 너무 많은 채찍질로 완벽함을 추구하는 최윤정 선생님-옆에서 많이 도와주지 못해 너무 미안한 맘이 듭니다, 몇 년을 하루같이 성실하게 생활하는 종열이, 언제나 비판적인 자세를 잃지 않는 진희, 실험을 함께했던 용우, 실험실 분위기를 화사하게 만드는 미란이, 말없이 열심히 하는 서연희 선생님과 재영이, 기초적인 염색과 섹션을 가르쳐주신 신영호 선생님께도 감사드립니다. 특히 마지막까지 라디에이터의 야릇한 조명아래 라꾸라꾸에서 동침하고, 뜨는 해를 바라보며 짧은 밤을 아쉬워했던 지희에게 매우 감사하며 아마도 같이 했던 시간들은 잊지 못할 것 같습니다.

또한, 실험적인 조언과 부족한 것들을 어디선가 구해주시는 옆 방 정현주 선생님, 유병기 선생님, 일상적인 문제들을 같이 고민해주시고 챙겨주시는 윤미혜 선생님께도 감사드립니다. 이 모든 분들이 있었기에 순간순간 포기하고 싶었던 긴 과정을 중단하지 않고 지속할 수 있었던 것 같습니다.

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Inflammation is a hall mark of various central nervous system (CNS) diseases and this is linked to glial activation. Microglia is the resident immune cells of CNS and is activated in response to brain injury and release neurotoxic factors including nitric oxide (NO) and proinflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β). Also, reactive gliosis is a component of an inflammatory process. It has been reported that agmatine given intraperitoneally to mice reduce the infarct area of brain from ischemic injury and possible neuroprotective mechanism. In this study, the role of agmatine on TNF-α, IL-1β, NO production in *in vitro* ischemia like injury model and in middle cerebral artery occlusion (MCAO) animal model were evaluated. To address the effect of agmatine on release of cytokines in cerebral ischemia, BV2 microglial cells were transferred

into an anaerobic chamber and treated with agmatine (100 μM) at the start of oxygen glucose deprivation (OGD) or lipopolysaccharide (LPS) (1 ㎍/㎖) treatment. MCAO was performed in male Sprague-Dawley (SD) rat for 90 minutes and agmatine (100 mg/kg) was administered by IP injection at the begining of and at the end of occlusion respectively. Agmatine treatment reduced cell death in microglial cells after OGD and LPS induced injury. It has been reported that this protection by agmatine is associated with decreased NOS activity and expression, as well as NO generation. Agmatine decreased the number of iNOS positive cells in MCAO and LPS induced injury model. iNOS immunoreactivity was also expressed in microglial cells and astrocytes, and the number of iNOS positive cells were increased in ipsilateral cerebral cortex and striatum after 24 hours from MCAO injury respectively.

Immunohistochemistry and Western blot analysis were also performed using antibodies of proinflammatory cytokines such as TNF-α, IL-1β, inducible nitric oxide synthase (iNOS), and nuclear factor-*k*B (NF-*k*B). The number of TNF-α and IL-1β positive cells were increased in ipsilateral cerebral cortex and striatum after MCAO or LPS injury, respectively, and agmatine treatment decreased the number of each cytokine immunopositive cells significantly. The number of NF-κB positive cells was also reduced in cerebral cortex and striatum after MCAO or LPS injection by agmatine treatment. Agmatine suppresses microglial activation, and the immunoreactivity for TNF-α, IL-1β, iNOS, NF-*k*B were predominantly reduced in agmatine treated group compared with non-treated group in microglia, but not in reactive astrocytes. This results show that agmatine suppressed the production of NO via inhibiting iNOS expression in microglia and reduced the expression of TNF-α and IL-1β through NF-κB translocation into nucleus during MCAO or LPS induced inflammatory injury. Thus, agmatine is suggested to have anti-inflammatory effect with suppression of microglial activation and NO production through regulation of NF-κB.

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key words : cerebral ischemia, inflammation, LPS, agmatine, iNOS, cytokine

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I. INTRODUCTION

Acute ischemic stroke is an important cause of disability and death in industrialized countries affecting up to 0.2 % of the population each year[1]. An inflammatory response occurs after stroke, and appears to contribute to ischemic brain injury. Brain inflammation is a hallmark of various CNS disease such as bacterial and viral infections, Alzheimer's disease, and cerebral ischemia, and involves the activation of microglia and astrocytes.

Microglia are considered as the resident immune cells of the central nervous system (CNS) and responsive to environmental stress and immunological challenges. In various neuropathologies, microglia are found to be activated by cytokines and injured or dead neuronal cells[2-7] and have been implicated as the predominant cell type governing inflammation-mediated neuronal damage. In particular, activated microglia release neurotoxic factors including inducible nitric oxide synthase (iNOS) and proinflammatory cytokines such as tumor necrosis

factor-α (TNF-α) and interleukin-1β (IL-1β). Studies using cell culture and animal models have demonstrated that excessive quantities of cytokines produced by activated microglia can be deleterious to neurons[8,9], and it is most certainly involved in potentiating ischemic damage[10,11].

Nitric oxide (NO) is known to trigger and mediate cascades involved in inflammation and apoptosis in ischemic injury and iNOS is also involved in the mechanisms by which ischemia-induced inflammation. iNOS is expressed predominantly in inflammatory cells infiltrating the ischemic brain. Excessive amount of NO derived from reactive astrocyte and microglia is assumed to contribute neuronal death during ischemia, trauma, and oligodendrocyte degeneration in demyelinating diseases. Also, the activity of iNOS is thought to be present mostly in microglia and macrophages. Studies related to iNOS indicated that induction of iNOS by a series of cytokines including TNF-α and IL-1β and interferon-ɣ (IFN-ɣ)[12], and may be in part responsible for the ischemic injury[13,14]. So, their inhibition is neuroprotective[15,16] and may be a useful therapeutic strategy to target selectively the progression of ischemic brain injury.

Nuclear factor-κB (NF-κB) is one of the most important transcription factors playing a pivotal role in mediating inflammatory responses to a variety of signals, including inflammatory cytokines, oxidative stress, hypoxia-reoxygenation, bacterial and viral infections, and ultraviolet light and irradiation[17]. Especially, NF-κB is also involved in acute phase of inflammatory responses that potentiate ischemic injury[18,19], activating many genes involved in the pathogenesis of cerebral ischemia, such as iNOS, TNF-α, IL-1β, intercellular cell adhesion molecule (ICAM)-1, cyclooxygenase-2 (COX-2), and IL-6[20]. And proinflammatory cytokines such as, TNF-α, IL-1β or IFN-ɣ bind to their respective receptors and induce iNOS expression via activation of NF-κB.

Agmatine, [$(NH_2(CH_2)_4NH_2C(NH)=NH$], a polycationic amine synthesized by decarboxylation of L-arginine by arginine decarboxylase (ADC), was first discovered in 1910. It is an endogenous clonidine-displacing

substance, an agonist for the α2-adrenergic and imidazoline receptors, and an antagonist at N-methyl-D-aspartate (NMDA) receptors[21-23]. It has been shown to exert some neuromodulatory functions in the central nervous system and have neuroprotective effect in trauma and neonatal ischemia models[24-28]. Recently, agmatine was shown to protect neurons against glutamate toxicity and this effect was mediated through NMDA receptor blockade, with agmatine interacting at a site located within the NMDA channel pore[29]. Despite this work, the mode and sites of action for agmatine in the brain have not been fully defined. Nitric oxide synthases (NOSs) generate nitric oxide (NO) by sequential oxidation of the guanidino group in L-arginine, and agmatine is an L-arginine analogue with a guanidino group. Being structurally similar to L-arginine, agmatine is also a competitive nitric oxide synthase (NOS) inhibitor[30,31]. This suggests that agmatine may protect the brain from ischemic injury by interfering with NO signaling.

The present study was aimed to evaluate whether agmatine modulate the ischemic neuroinflammation in both *in vitro* using BV2 microglial cell line and *in vivo* against transient focal cerebral ischemia in adult rat.

II. MATERIALS AND METHODS

1. *In Vitro* Model

A. BV2 cell culture and *lipopolysaccharide* (LPS) treatment

The immortalized murine BV2 cell line that exhibits both the phenotypic and functional properties of reactive microglial cells[32] were grown and maintained in Dulbecco's modified Eagle's medium supplemented with 10 % fetal bovine serum (Hyclone), streptomycin, and penicillin (Hyclone). For experiments, cells were plated into 24-well plates at a density of 4×10^5 cells/well. After 24 hours incubation, cells were washed twice with warm DMEM and then treated in serum-free medium. In all experiments, cells were treated with LPS (1 ㎍/㎖) alone or with agmatine simultaneously (co-treatment) or 2 hours after LPS treatment (post-treatment). Agmatine was dissolved in PBS, and its concentration is 100 μM.

B. Oxygen-glucose deprivation (OGD)

Cultures were transferred to an anaerobic chamber (Forma Scientific Co.) (O_2 tension < 0.2 %), washed in deoxygenated, serum-free DMEM, and incubated at 37 ℃ for 2 hours. OGD was ended by returning the cultures to the normoxic incubator for 20 hours. Agmatine (100 μM, Sigma) was added to the culture medium at the start of injury (CO treatment group, CO), and at reperfusion when oxygen were restored (Post treatment group, POST). Preliminary studies tested agmatine concentrations from 10 to 300 μM. Cell death was reduced at concentrations of 100 μM and greater so we used 100 μM for subsequent experiments. Injury was assessed at the end of reperfusion.

C. Evaluation of cell death

(A) Measurement of lactate dehydrogenase (LDH) activity

Cell lysis was quantified by assay of lactate dehydrogenase (LDH) released into the culture medium[33] after OGD for 2 hours and reperfusion for 20 hours. The amount of total LDH released of 100 % cell death named "full kill" was determined at the end of experiment following freezing at -70 ℃ and rapid thawing. The extent of cell death was expressed as percentage of full kill.

(B) Hoechst-PI nuclear staining

Cell death was also evaluated morphologically by staining of non-viable cells with propidium iodide (Sigma, St. Louis, Missouri, USA), and living cells stained with Hoechst 33258 dye (Sigma, St. Louis, Missouri, USA). Staining with the fluorescent dyes propidium iodide and Hoechst 33258 allows discrimination of apoptotic from non-apoptotic cells on the basis of nuclear morphology and evaluation of membrane integrity. Hoechst dyes added to the culture medium (final concentration of 2-5 ㎍/㎖) and then cells were kept at 37 ℃ for 30 minutes. Propidium iodide solution was then added (final concentration 2-5 ㎍/㎖) just before observation in a Olympus microscope equipped for epifluorescence with a UV filter block.

D. Concentration of Nitrite (NO_2^-) and Nitrate (NO_3^-)

To assess NO production, the measurement of the stable end-products of NO metabolism, nitrite (NO_2) and nitrate (NO_3) were measured in medium from cultures using the Griess reaction (Sigma, St. Louis, Missouri, USA)[34,35]. In brief, 100 ㎕ of media were added to 96 well plate in duplicate. 100 ㎕ of Griess reagent was added to each well. The plate was mixed gently, and allowed to incubate in dark room for 15 minutes at room temperature. The absorbance of the reaction product was measured at 540 nm on a microplate reader. Each experiment was repeated over 12 times with cells from 5 different preparation.

E. Immunoblotting

Expression of TNF-α, IL-1β, iNOS and NF-κB proteins was estimated by immunoblotting in ipsilateral cerebral hemisphere at 24 hours after MCAO injury and LPS microinjection. Cells cultured in 10 cm Petri-dishes were washed twice with ice-cold PBS and lysed in 300 ㎖ lysis buffer (50 mM Hepes, pH 7.5, 100 mM NaCl, 2 mM EDTA, 10 % glycerol, 1 % Triton X-100, 3 mM benzamidine, 1 mM sodium pyrophosphate, 10 mM sodium orthovanadate, 100 mM NaF, 1 mM PMSF, 10 ㎎/㎖ aprotinin, 10 ㎎/㎖ leupeptin, and 5 ㎎/㎖ pepstain A). After incubation for 30 minutes on ice, cell lysates were centrifuged (13,000 rpm, 15 minutes, 4 ℃), and the supernatants were recovered. The protein concentration of the samples was determined by the Bradford assay (Bio-Rad, Hemel, Hempstead, UK) and samples were equilibrated to 2 ㎎/㎖ with lysis buffer. Cell extracts were prepared and 100 ㎍ of proteins were separated by SDS-PAGE gel electrophoresis and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 5 % bovine serum albumin in 10 mM Tris. HCl containing 150 mM NaCl and 0.5 % Tween 20 (TBST) and then incubated with primary antibodies that recognize TNF-α (1:1000, Santa Cruz, CA), IL-1β (1:1000, Santa Cruz, CA), iNOS (1:1000, Chemicon, ML, USA), NF-κB (1:2000, Chemicon, ML, USA) and actin (1:1000, Santa cruz, CA). After thoroughly washing with TBST, horseradish peroxidase-conjugated secondary antibodies (New England Biolabs, Beverly, MA; 1:3000 dilution in TBST) were applied and the blots were developed using an chemiluminescence detection kit (Amersham, Piscataway, NJ, USA). The bands were visualized with the ECL detection system using Kodak X-ray film[36].

F. Measurement of cytokine levels by enzyme-linked immunosorbent assay (ELISA)

For the cytokine immunoassay, the BV2 cells (2×10^5 cells/㎖) were cultured in 24-well plates, and stimulated with LPS (1 ㎍/㎖) for 22 hours with or

without agmatine (100 mM). The supernatants of the BV2 cells were collected at set times (1 hr, 3 hrs, 24 hrs) and the concentrations of TNF-α, IL-lβ were measured by ELISA using monoclonal antibodies and the procedure recommended by supplier (E-bioscience, San Diego, CA, USA).

2. In vivo Model

A. Animals

Male Sprague-Dawley rats weighing between 270 and 300 g were purchased from Samtako (osan, ROK). All animal procedures were carried out according to a protocol approved by the Yonsei University Animal Care and Use Committee in accordance with NIH guidelines. Rats were kept on a 12 hr light/dark cycle with ad libitum access to food and water. Rats were acclimated to their environment for 3 days before use for experiments.

B. Focal Cerebral Ischemia Model

Male rats weighing 270 - 300 g were subjected to transient middle cerebral artery occlusion (MCAO, n=50). Animals were anesthetized with ketamine cocktail. Depth of anesthesia was assessed by toe pinch every 15 minutes. Rectal temperature, respirations, heart rate were monitored and maintained in the physiologic range throughout the surgery. Focal cerebral ischemia was induced using an occluding intraluminal suture[37]. The left carotid artery and its branches were exposed through a midline cervical incision. The left external carotid artery (ECA) was tied by a 3-0 monofilament nylon and blocked its branches. A piece of uncoated 3-0 Dermalon suture (blue monofilament nylon DG, Ethicon, Johnson-Johnson, Somerville, NJ), with its tip rounded by gentle heating was inserted into the arteriotomy and advanced under direct visualization and gently advanced 17.5 ㎜ into the internal carotid artery (ICA) from the bifurcation to occlude the ostium of MCAO

until rCBF was reduced to 15 - 20 % of the baseline (recorded by laser doppler flowmeter). After 90 min of occlusion, the suture was pulled back to restore the blood flow (confirmed by the return of rCBF to the baseline level), the site on arteriotomy was tied and surgical incisions were closed. Animals were allowed to recover. Twenty-two hours later, animals were sacrificed.

C. Measurement of cerebral blood flow (CBF)

A rat was placed in a stereotaxic frame fitted. The craniectomy (3 ㎜ in diameter, 6 ㎜ lateral and 2 ㎜ caudal to bregma) was performed with extreme care over the middle cerebral artery territory using a trephine. The dura was left intact and the probe was placed over the brain in a region corresponding to the ischemic core and fixed to the periosteum. The probe was connected to a laser flowmeter device (Omega flow, FLO-C1, Neuroscience, Tokyo, Japan) for continuous monitoring of regional cerebral blood flow (rCBF). Stable readings were obtained before advancing the suture, and relative CBF (rCBF) was determined using Laser-Doppler Flowmetry (Omega flow, FLO-C1, Neuroscience, Tokyo, Japan) and expressed as the % baseline. rCBF was estimated at four different times : before and during MCAO (90 min), and 1 hr and 2 hours after reperfusion. Blood flow measurements were performed on 3 animals in the CO and EC groups[38,39]

D. Agmatine administration

Agmatine was dissolved in normal saline (100 ㎎/㎏, Tocris) and given intraperitoneally at the time of occlusion (CO, n=12), or at the end of occlusion after the suture was removed (POST 0h, n=12). Experimental controls received normal saline in equivalent volumes (EC).

E. Microinjection of LPS into corpus callosum

Rats were anesthetized with ketamine cocktail (300 ㎎/㎏) and positioned in a small-animal stereotaxic apparatus (David Kopf Instruments, Tujunga, CA) to conform to the brain atlas. Microinjection of LPS (*Escherichia coli* stereotype 055:B5; Sigma, St. Louis, MO, USA) into the corpus callosum was performed with a 32-gauge needle through a dentist's burr hole. To perform the injection into the corpus callosum, the following coordinates were used: 0.1 ㎜ posterior from bregma, 1.8 ㎜ lateral from the sagittal suture, and 3.2 ㎜ below the dura mater. LPS (1 ㎍/㎕) or vehicle injection (equivalent volume of sterilized saline) were performed.

F. Histological Examination

Rats were anesthetized with chloral hydrate and decapitated at 24 hours after MCAO and LPS microinjection. Rat brains were cut into coronal slices of 3 ㎜ in thickness using a rat brain matrix (Ted Pella, Redding, CA, USA). The brain slices were fixed with 4 % paraformaldehyde (pH 7.4) in 0.1 M phosphate buffer (PB) for 1 day and subsequently embedded in paraffin block. After paraffin embedding, 5 ㎛ thick sections were stained with Hematoxylin and Eosin (H&E) (Muto chemicals, Tokyo, Japan) and immunostained[36].

G. Immunohistochemistry

Brain sections were deparaffinized and rehydrated. Sections were immunostained with antibodies against OX-42 (1:500 Abchem, Cambridge, MA) iNOS (1:1000, Chemicon, CA), TNF-α (1:500, Santa Cruz, CA), IL-1β (1:500, Santa Cruz, CA), and NF-κB (1:500, Chemicon, CA) followed by an appropriate biotinylated secondary antibody. Stains were visualized using the ABC kit (Vector, CA), then reacted with diaminobenzidine (DAB) (Zymed, San Francisco, CA). Immunostaining controls were prepared by incubating tissues without primary antibodies. All incubation steps were performed in a

humidified chamber.

H. Immunoblotting

Expression of TNF-α, IL-1β, iNOS and NF-κB proteins was estimated by immunoblotting in ipsilateral cerebral hemisphere at 24 hours after MCAO injury and LPS microinjection. The injured tissue were prepared by homogenization, and 50 ㎍ of proteins were separated by SDS-PAGE gel electrophoresis and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA). The membranes were blocked with 5 % bovine serum albumin in 10 mM Tris. HCl containing 150 mM NaCl and 0.5 % Tween 20 (TBST) and then incubated with primary antibodies (1:1000) that recognize TNF-α (Santa Cruz, Santa Cruz, CA), IL-1β (Santa Cruz, Santa Cruz, CA), iNOS (Chemicon, ML, USA), NF-κB (Chemicon, ML, USA) and actin (Santa Cruz, Santa Cruz, CA, USA). After thoroughly washing with TBST, horseradish peroxidase-conjugated secondary antibodies (New England Biolabs, Beverly, MA; 1:3000 dilution in TBST) were applied and the blots were developed using an chemiluminescence detection kit (Amersham, Piscataway, NJ). The bands were visualized with the ECL detection system using Kodak X-ray film[36].

I. Statistical analysis

Statistical tests to determine differences between groups were performed with student's t test using SPSS ver 13.0 (SPSS, Chicago, IL, USA). P value 〈0.05 was considered significant. Data are expressed as the mean±standard deviation (SD).

III. RESULTS

1. Protective effect of agmatine on BV2 microglial cells under ischemia like injury or LPS induced inflammatory injury

A. Measurement of LDH assay

To investigate the influence of agmatine on cell death of microglial cells, cultured BV2 cells were transferred to anaerobic chamber for 2 hrs with or without agmatine (100 μM). LDH release (%) was measured to know the degree of cell death under two different agmatine treatment conditions. LDH release in agmatine co-treatment group was 41.02±4.3 %, and that in post-treatment group was 34.99±4.32 %. LDH release in agmatine co-treatment group reduced by 25.8 % compared to that in OGD injured experimental control group by LDH release assay. (Figure 1A)

On the other hand, cytotoxicity in LPS (1 ㎍/㎖) - stimulated BV2 microglia was also determined by measuring the LDH release (%). LDH release in agmatine co-treatment group was 43.15±5.03 %, and that in agmatine post-treatment group was 23.7±2.3 %. Agmatine co-treatment reduced LPS induced injury by 26.2 % compared to experimental control group by LDH release (Figure 1B).

B. Hoechst-PI nuclear staining

Hoechst-PI staining was performed to assess cell death morphologically. Cultured BV2 cells were transferred to anaerobic chamber for 2 hrs or treated with 1 ㎍/㎖ LPS for 24 hrs. Non-viable cells stained with propidium iodide were detected as red color and live cells stained with Hoechst 33258 dye were detected as blue color. Only the agmatine co-treatment group (CO) had many live cells compared to cultures without agmatine treatment group (EC). The most of BV2 cells showed distinctive mophological feature of apoptosis - nuclear condensation and fragmentation into apoptotic bodies under OGD injury (Figure 2A) and LPS induced

injury (Figure 2B).

(A) (B)

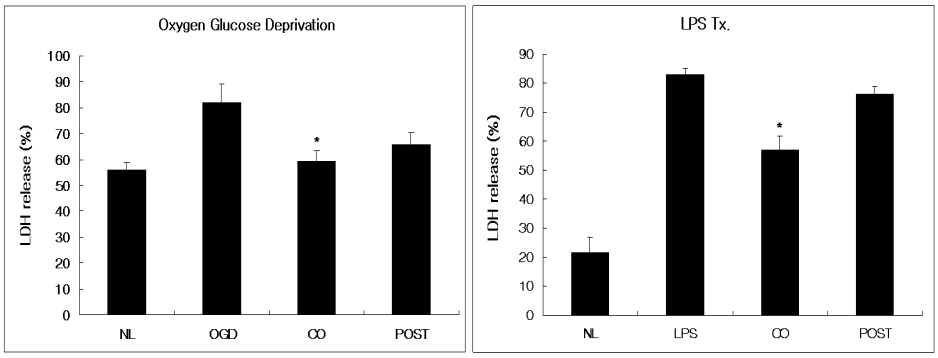


Figure 1. Effect of agmatine (100 μM) on the BV2 cells at 20 hrs after ischemia-like injury (A), and LPS induced injury (B) by LDH release. LDH release (%) was assayed for relative total cellular death. Data are expressed as mean±SEM. Co-treatment agmatine group (CO) significantly reduced the LDH release compared to non-treatment control group (EC) by student t-test (*: $p < 0.001$).

C. NO production

In order to evaluate the anti-inflammatory effects of agmatine in inflammatory BV2 microglia, nitric oxide (NO) production were measured in medium from cultures after OGD injury and LPS treatment respectively. After OGD injury for 24 hrs, the supernatants were taken, and the amount of NO was measured. Compared to normoxic cells, OGD and reoxygenation markedly induced NO production in BV2 cells. The nitrite and nitrate concentration in the media of the OGD injurry group was 9.43±0.73 μM, and that of the co-treatment group was 3.75±0.42 μM, and that in post-treatment group was 5.31±0.68 μM (Figure 3A).

Exposure to LPS enhanced the expression of NO in BV2 microglia. Amount of NO in OGD injured group was 16.4±3.14 μM, and that of the co-treatment group was 5.5±1.27 μM, and that in post-treatment group was

10.66±1.44 μM (Figure 3B). The result showed that agmatine significantly suppressed NO production in BV2 cells compared to each experimental control group after OGD and LPS treatment respectively.

D. Expression of iNOS protein

Western blot analysis executed whether the inhibitory effect of agmatine on NO production was associated with decreased iNOS expression in oxygen glucose deprivated or LPS-stimulated BV2 microglia.

In OGD injury, the expression of iNOS was increased in cytosolic fraction of experimental control group compared to that in agmatine co-treatment group at 22 hrs after reoxygenation (Figure 4A)

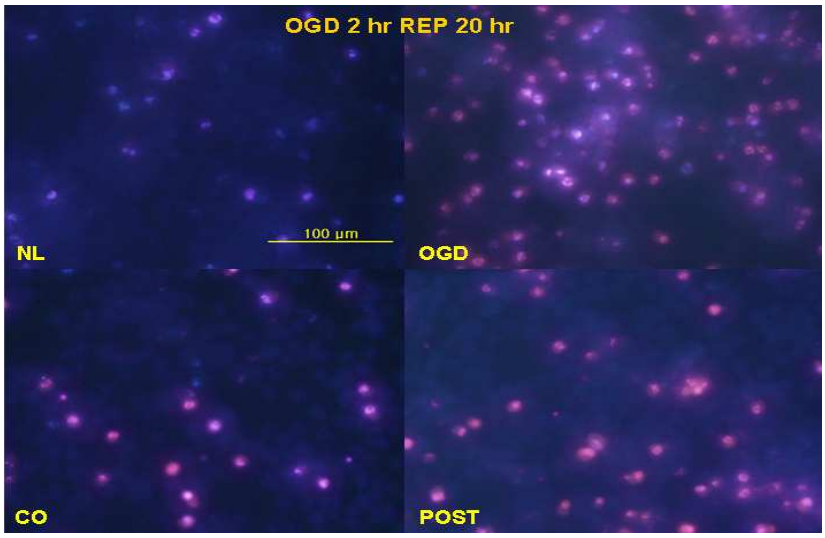
The expression of iNOS in BV2 cells after LPS (1㎍/㎖) treatment was increased in experimental control group but agmatine markedly attenuated the expression of iNOS at protein level (Figure 4B).

E. Production of cytokines

Enzyme linked immunosorbent assay (ELISA) was performed to elucidate the potential effects of agmatine on LPS-induced microglia activation in BV2 cells stimulated with LPS (1 ㎍/㎖) for 24 hours in the presence or absence of agmatine (100 mM). On the other hand, MG132, which is a NF-κB inhibitor was treated at the same time to demonstrate that inhibition of agmatine in LPS-induced production of cytokine through NF-κB activation.

The results show that LPS increased the production of concentration of TNF-α and IL-1β (basal level: 374.4 pg/ml) by 5.3- and 3.16-fold at 1hr, respectively. Agmatine co-treatment effectively decreased LPS-induced production of TNF-α and IL-1β to 1359.3 pg/ml and 640.57 pg/ml, respectively (Figure 5). As shown in Fig. 5, MG132 significantly reduced LPS-induced production of TNF-α and IL-1β to 1215.3 pg/ml and 305.4 pg/ml, respectively.

(A)



(B)

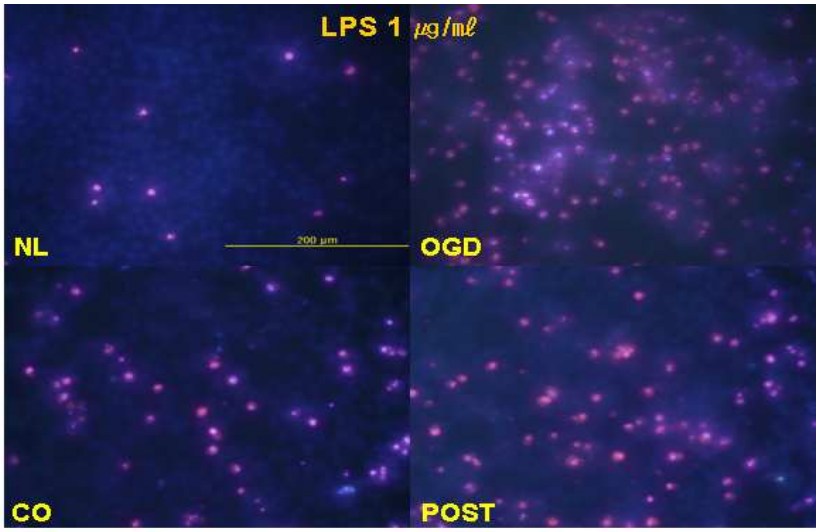


Figure 2. The photographs of BV2 cells stained with Hoechst-propidium iodide. BV2 cells incubated 24 hrs after ischemia-like injury (OGD) (A) or LPS (1 ㎍/㎖) stimulated injury (B) with or without agmatine (100 μM). Apoptotic cell death was observed in injured group, but the degree of cell death decreased in agmatine co-treatment group (CO) compared to experimental control group (EC). ×200

(A) (B)

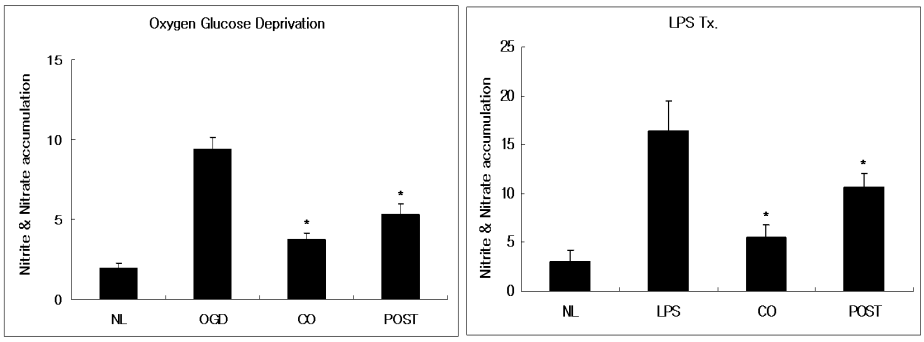


Figure 3. Concentration of Nitrite (NO_2^-) and Nitrate (NO_3^-) in the BV2 cells at 24 hrs after ischemia-like injury (OGD) (A) or LPS (1 ㎍/㎖) stimulation (B) with or without agmatine (100 uM). Data are expressed as mean±SEM. NO generation in co-treatment group (CO) significantly decreased compared to non-treatment group (EC) by student t-test (*: $p < 0.001$).

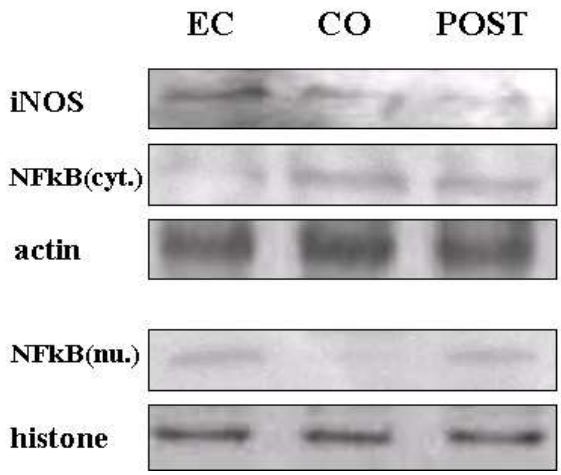
F. Activation of NF-κB

Since the activation of NF-κB by LPS can induce expression of proinflammatory mediators, NF-κB activity in OGD injury and LPS stimulated injury was verified using Western blot analysis.

In OGD injury, the expression of NF-κB was increased in nuclear fraction of experimental control group compared to that in agmatine co-treatment group at 22 hrs after reoxygenation, but the expression of NF-κB in nuclear fraction was reduced by agmatine treatment (Figure 4).

As shown in Fig. 6, the expression of nuclear NF-κB in BV2 cells after LPS (1㎍/㎖) treament was increased but, agmatine co-treatment significantly attenuated this nuclear translocation.

(A)



(B)

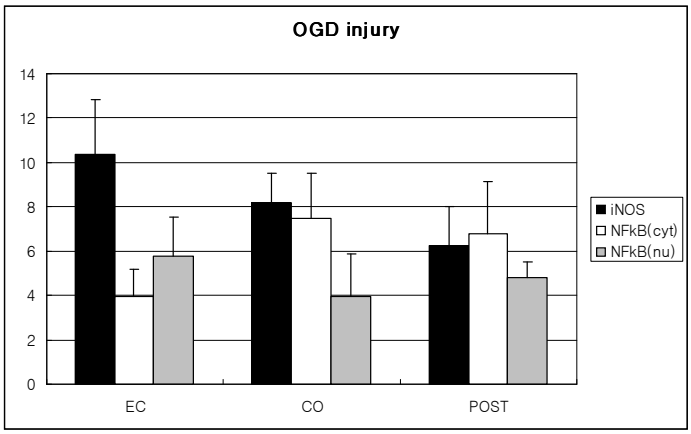
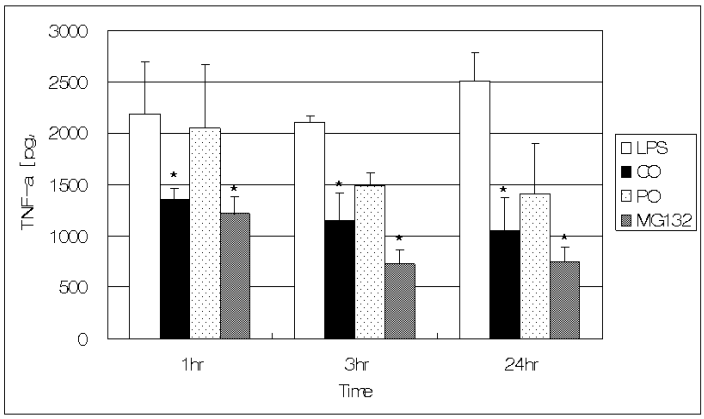


Figure 4. The expression of iNOS and NF-κB in BV2 cells at 24 hrs after OGD injury. The expression of iNOS was increased in experimental control group but agmatine decreased the expression of iNOS at protein level. The expression of NF-κB was increased in cytosolic fraction by agmatine treatment and that of nuclear NF-κB by agmatine was decreased compared to EC group. Relative optical densities (ROD) of iNOS are expressed as the band density compared to the density of β-actin. EC, Experimental control group; CO, Agmatine co-treatment group; POST, Agmatine post-treatment group.

(A)



(B)

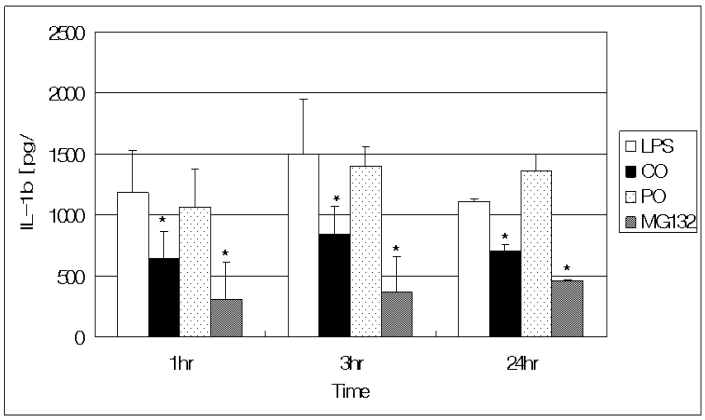
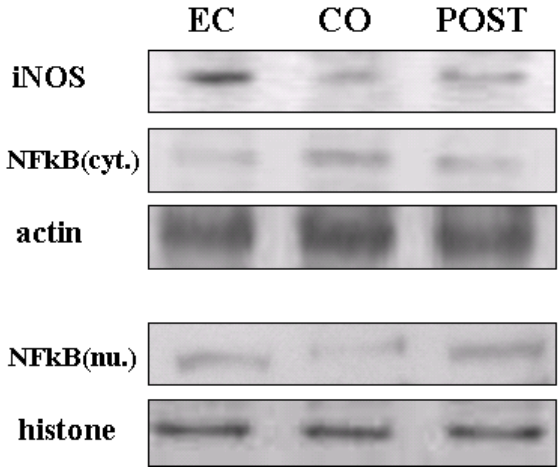


Figure 5. Effect of agmatine on production of proinflammatory cytokines in LPS-stimulated BV2 microglia. Cells were stimulated with LPS (1 ㎍/㎖) for 24 hrs with or without agmatine (100 mM) and the supernatants were collected at 1 hr, 3 hrs, and 24 hrs after LPS treatment, respectively. The amounts of TNF-α (A) and IL-1β (B) in the supernatant were measured by ELISA. The secretion of TNF-α and IL-1β were increased in experimental control group (EC) but agmatine decreased that of TNF-α and IL-1β compared to EC group. Each value indicates the mean±S.E.M. and is representative of results obtained from three independent experiments. *$P < 0.05$ are significantly different from the value in cells treated with LPS in the absence of agmatine.

(A)



(B)

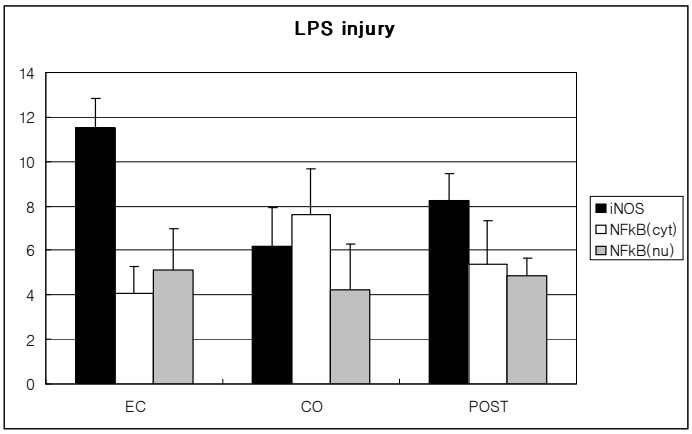


Figure 6. The expression of iNOS and NF-κB in BV2 cells after 24 hours LPS (1㎍/㎖) treatment. The expression of iNOS was increased in cytosolic fraction by LPS stimulation. The expression of nuclear NF-κB was decreased compared to EC group by agmatine. Relative optical densities (ROD) of iNOS, TNF-α, IL-1β, and NF-κB are expressed as the band density compared to the density of β-actin. EC, Experimental control group; CO, Agmatine co-treatment group; POST, Agmatine post-treatment group.

2. Anti-inflammatory effect of agmatine in *in vivo* animal model

A. Transient MCAO model

(A) Histological analysis for anti-inflammatory effect of agmatine after MCAO injury

After transient MCAO, the tissue of ischemic core was all destroyed (data not shown), but the myriad of microglia, gliosis, vacuolization were observed in penumbra area of experimental control group, but the gliosis and microglial activation reduced in agmatine co-treatment group following hematoxyline-eosin staining (Figure 7). Activated microglia, as identified by their enlarged size, shorter and thicker processes, were stained intensely by OX-42 in experimental control group. Also, the number of OX-42 positive cells increased in ipsilateral cerebral cortex of penumbra and striatum, but it was significantly decreased in agmatine co-treatment group after MCAO brain injury (Figure 8).

(B) Immunohistochemical analysis

TNF-α, IL-1β, iNOS immunopositive cells with elongated cell bodies and one or more thick and short processes were observed under ischemic penumbra. As shown in Fig. 9, iNOS immunoreactivity was expressed in microglial cells and astrocytes, and the number of iNOS positive cells was increased in ipsilateral cerebral cortex and striatum after 24 hours from MCAO injury respectively, but the number of iNOS positive cells was reduced in ipsilateral cerebral cortex and striatum by agmatine treatment. In similar to iNOS immunohistochemical result, the number of TNF-α and IL-1β positive cells were increased in ipsilateral cerebral cortex and striatum after 24 hours from MCAO injury respectively, and agmatine treatment decreased the number of every cytokine immunopositive cells significantly in the ipsilateral

striatum (Figure 10 & 11).

(C) Expression of proinflammatory cytokine

Western blot analysis was performed using antibodies against TNF-α, IL-1β, iNOS, and NF-κB. In penumbra area, the expression of TNF-α, IL-1β, and iNOS was increased in cytosolic fraction of experimental control group compared to that in agmatine treatment group at 24 hours after MCAO. The expression of NF-κB in nuclear fraction was reduced by agmatine treatment (Figure 12).

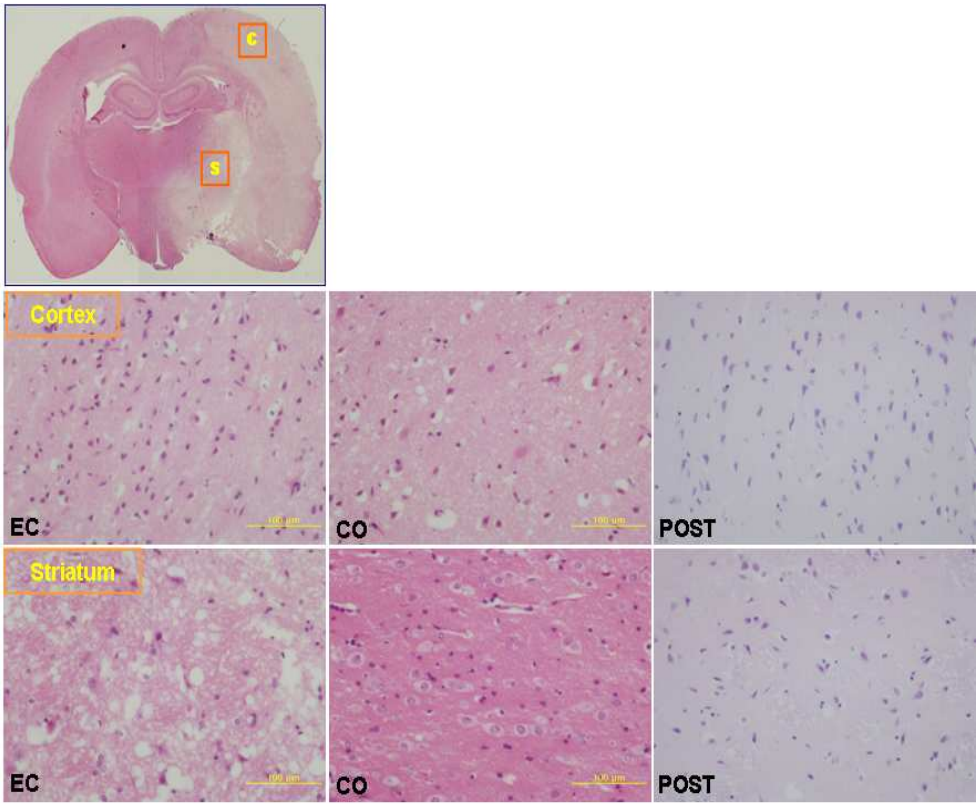
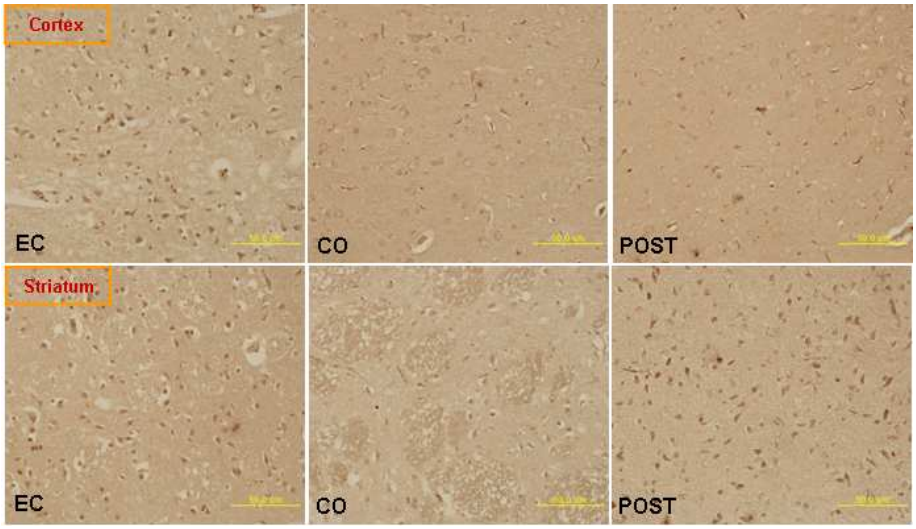


Figure 7. H&E staining of ipsilateral cortical penumbra and striatum after 24 hrs in MCAO injury model. Red neurons and vacuolization were observed in experimental control group (EC) at 24 hrs after MCAO, but the gliosis and microglial activation reduced in agmatine co-treatment group (CO).

(A)



(B)

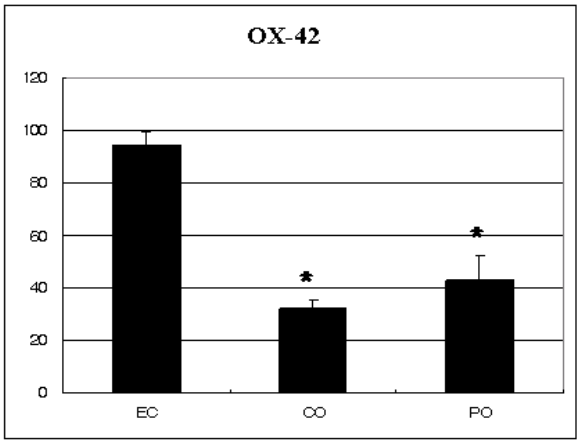
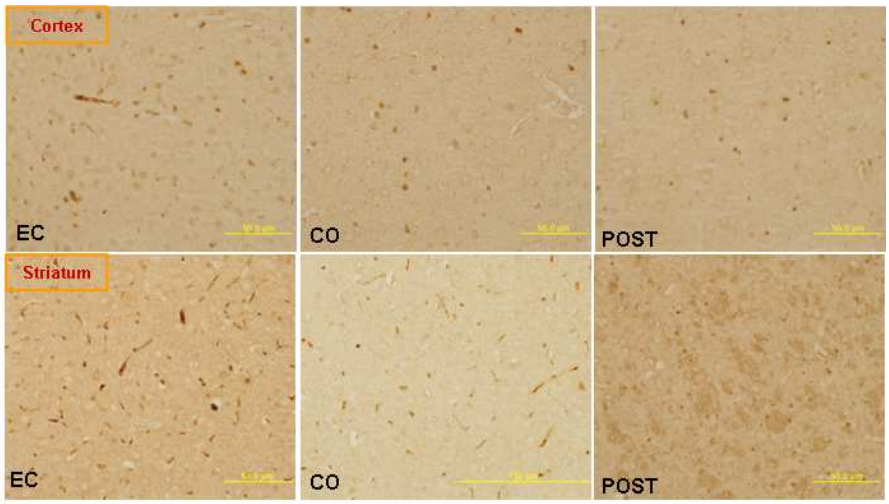


Fig 8. Immunohistochemical study for OX-42 in the ipsilateral penumbra at 24 hrs after MCAO injury. OX-42 positive cells were stained in microglial cells and blood vessels (A). The number of OX-42 positive glial cells in the ipsilateral cerebral cortex and striatum was increased in experimental control group (EC), but it was significantly decreased in agmatine (100 ㎎/㎏) co-treatment group (CO) at 24 hrs after MCAO injury.

(A)



(B)

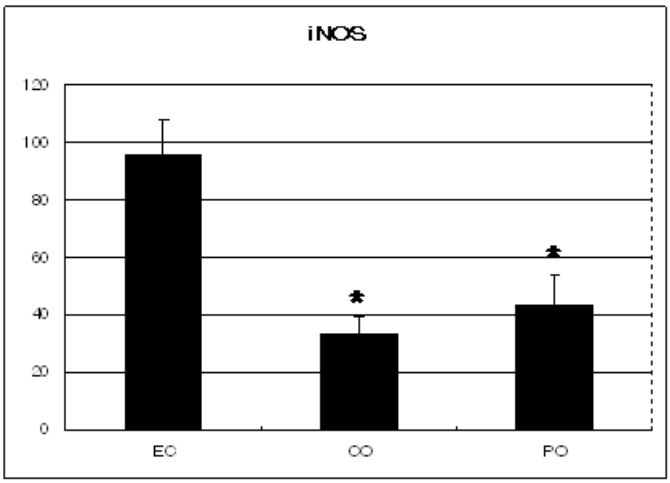
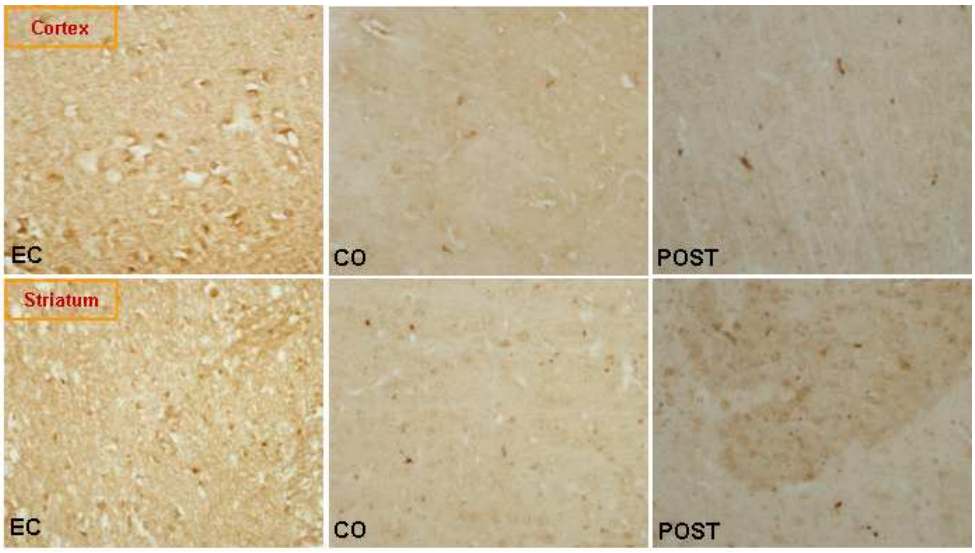


Fig 9. Immunohistochemical study for iNOS in the ipsilateral penumbra at 24 hrs after MCAO injury. The number of iNOS positive glial cells were increased in experimental control group (EC), but it was effectively decreased in agmatine (100 ㎎/㎏) co-treatment group (CO) at 24 hrs after MCAO brain injury (A, B).

(A)



(B)

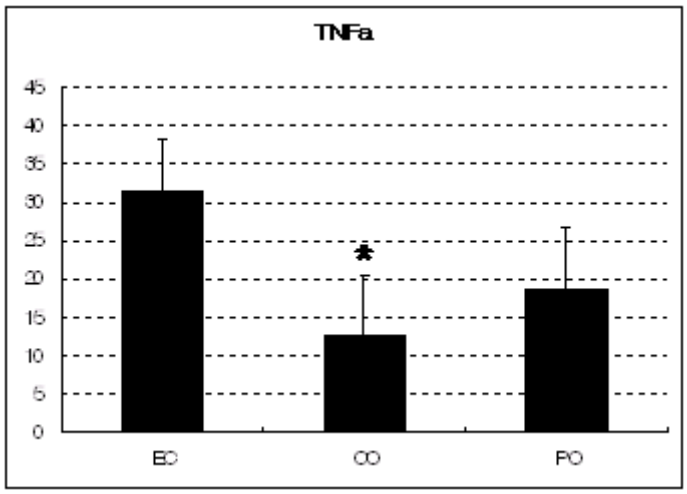
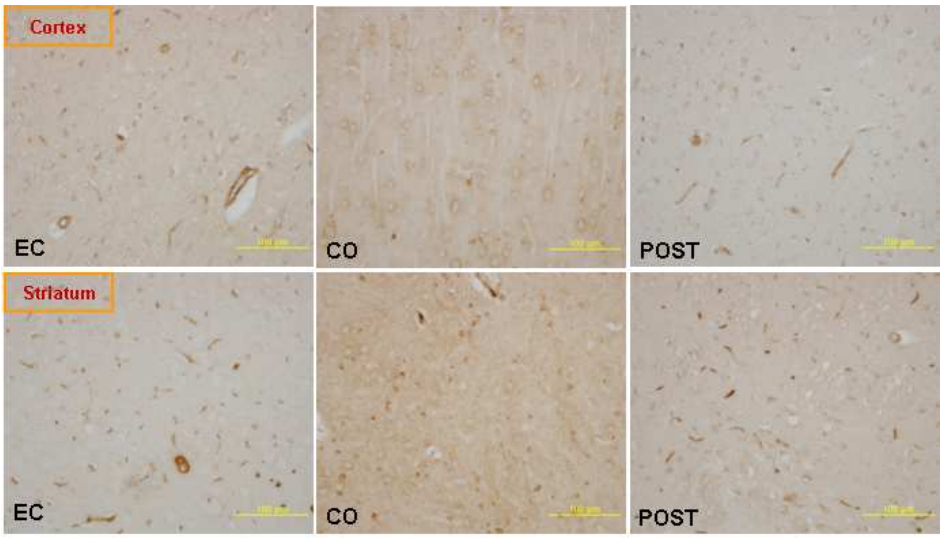


Fig 10. Immunohistochemical study for TNF-α in the ipsilateral penumbra at 24 hrs after MCAO injury. TNF-α positive cells were expressed in microglial cells in ipsilateral penumbra (A). The number of TNF-α positive glial cells was increased in experimental control group (EC), but it was significantly decreased in agmatine (100 ㎎/㎏) co-treatment group (CO) (B).

(A)



(B)

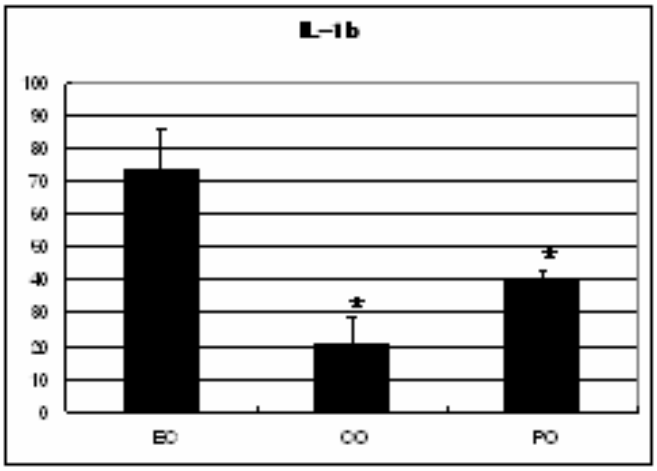
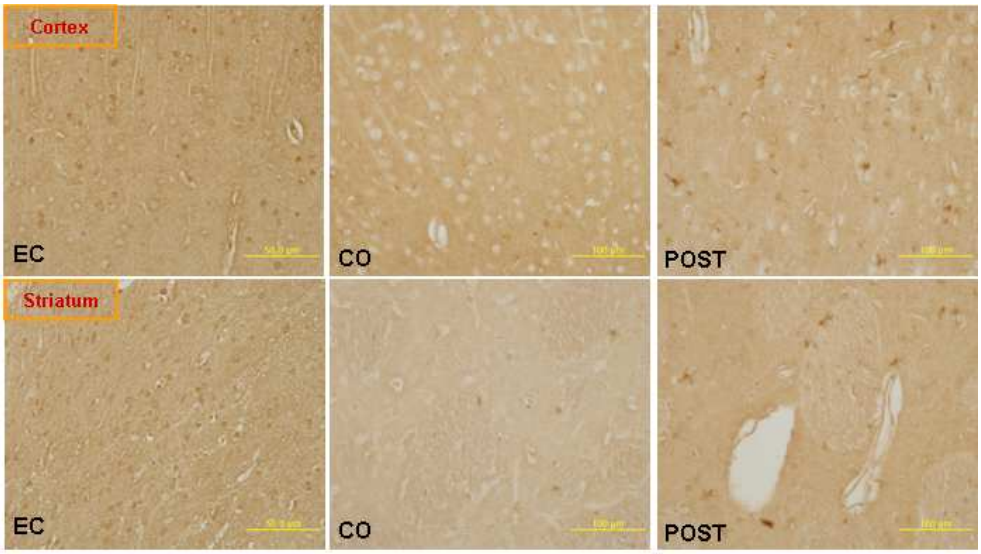


Fig 11. Immunohistochemical study for IL-1β in the ipsilateral penumbra at 24 hrs after MCAO injury. IL-1β positive cells were expressed in microglial cells and blood vessels after 24 hrs from MCAO injury (A). The number of IL-1β positive glial cells in ipsilateral cerebral cortex and striatum were increased in experimental control group (EC), but it was significantly reduced in agmatine (100 ㎎/㎏) co-treatment group (CO) (B).

(A)



(B)

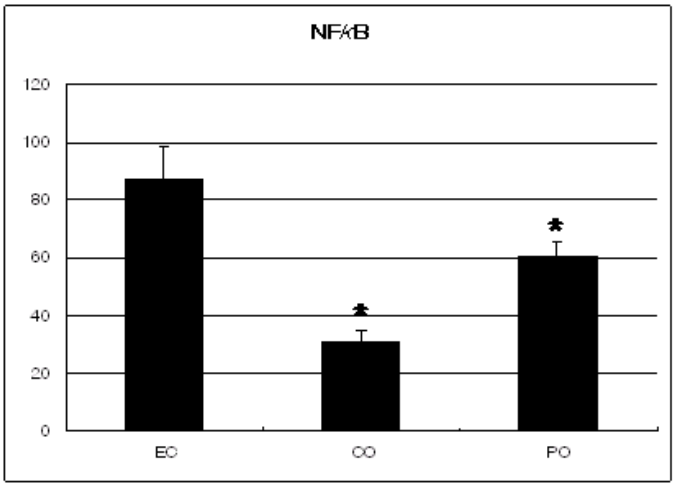
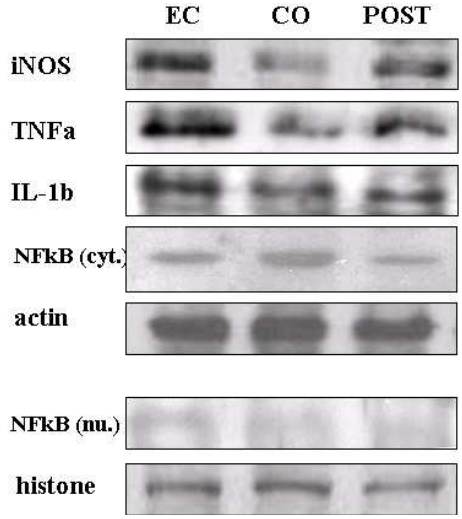


Fig 12. Immunohistochemical study for NF-κB in the ipsilateral penumbra at 24 hrs after MCAO injury. The number of NF-κB positive glial cells were increased in experimental control group (EC), but it was significantly decreased in agmatine (100 ㎎/㎏) co-treatment group (CO) (B).

(A)



(B)

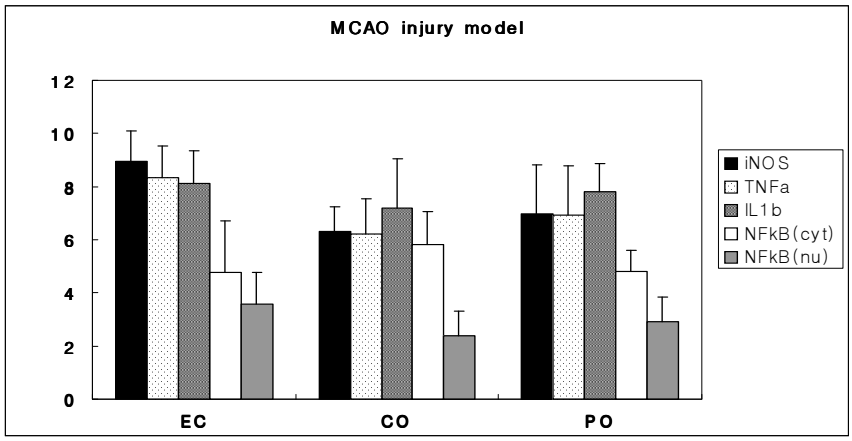


Fig. 13. The expression of iNOS, TNF-α, IL-1β, and NF-κB in rat brain after 24 hrs from transient MCAO. In ipsilateral cerebral cortex, expression of iNOS, TNF-α, and IL-1β was increased, but agmatine reduced the expression of iNOS, TNF-α, and IL-1β. The expression of nuclear NF-κB was increased in EC group compared to agmatine co-treatment group. Relative optical densities (ROD) of iNOS, TNF-α, IL-1 β, and NF-κB are expressed as the band density compared to the density of β actin. EC, Experimental control group; CO, Agmatine co-treatment group; POST, Agmatine post-treatment group.

B. LPS intracerebral microinjection model

We investigated the effect of agmatine on inflammatory cells in LPS stimulated brain.

(A) Histological analysis for anti-inflammatory effect of agmatine on LPS induction

The effect of agmatine was shown by coronal sectioned brain with H&E staining. After 24 hours from LPS intracerebral microinjection, the gliosis, necrotic neurons were observed and the number of microglia increased in experimental control group, but the gliosis and microglial activation were reduced in agmatine co-treatment group following hematoxylin-eosin (H&E) staining (figure 14). In immunohistochemical staining of OX-42, which is microglial marker, OX-42 immunoreactivity was represented in microglial cells and macrophages in blood vessels, and shorter and thicker processes was observed as compared to the non-activated state. Also, the number of OX-42 positive cells increased in ipsilateral cerebral cortex, but it was significantly decreased in agmatine co-treatment group after LPS treatment.

(B) Identification of cytokine expressing cells

In LPS treatment group, the number of iNOS positive cells was increased in ipsilateral cerebral cortex and striatum after 24 hours LPS injection, but that of iNOS positive cells reduced in ipsilateral cerebral cortex and striatum by agmatine treatment (Figure 15A). Also, iNOS positive cells were overlapped OX-42 positive cells in LPS stimulated inflammatory brain (Figure 15B)

In similar to iNOS result, agmatine treatment decreased TNF-α immunoreactivity significantly in ipsilateral hemisphere at 24 hours after LPS stimulation compared to experimental control group (Figure 16).

Experimental control group showed extensive increase of IL-1β immunopositive cells in both ipsilateral cerebral cortex and striatum, while agmatine co-treatment group demonstrated only minor immunoreactivity after 24 hours from LPS injection (Figure 17).

The number of NF-κB positive cells was increased respectively in ipsilateral cerebral cortex and striatum at 24 hours after LPS injection, but that of NF-κB positive cells reduced in ipsilateral cerebral cortex and striatum by agmatine treatment (Figure 18).

TNF-α and IL-1β immunoreactivity expressed in small and rod shape cells were identified in double-labelling study with activated microglial cells (Figure 16B & 17B).

(C) Expression of proinflammatory mediators, and NF-κB

Western blot analysis was performed using antibodies against TNF-α, IL-1β, iNOS, and NF-κB. In ipsilateral cerebral cortex, the expression of TNF-α, IL-1β, and iNOS were increased in experimental control group compared to that in agmatine co-treatment group at 24 hours after LPS intracerebral microinjection. On the other hand, the expression of nuclear NF-κB was increased in experimental control group, agmatine attenuated the expression of NF-κB in nuclear fraction at 24 hours after LPS (Figure 19).

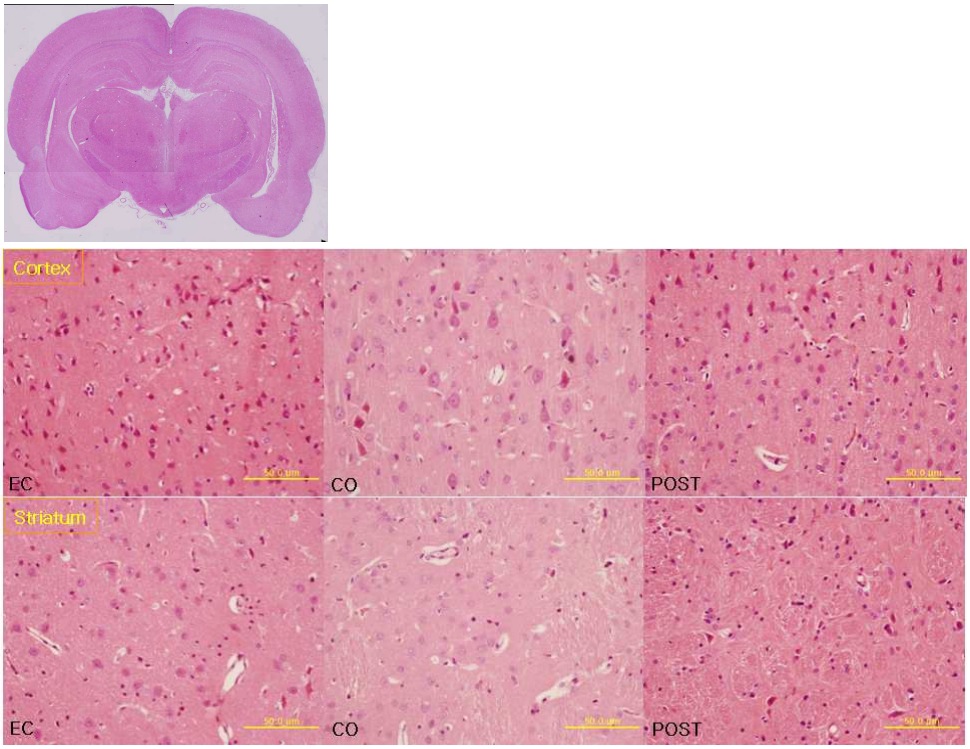
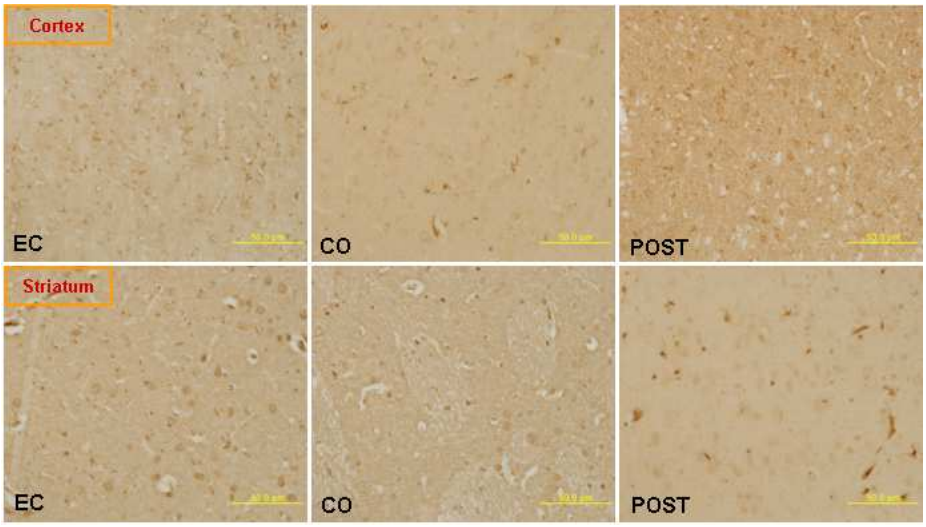


Fig 14. The effect of agmatine in LPS microinjection model was shown by coronal sectioned cerebral hemisphere with H&E staining. After 24 hours from LPS intracerebral microinjection, red neurons were observed and the number of microglia increased in experimental control group (EC), but microglial activation decreased and normal cytoarchitecture was shown in agmatine co-treatment group (CO) following hematoxyline-eosin staining.

(A)



(B)

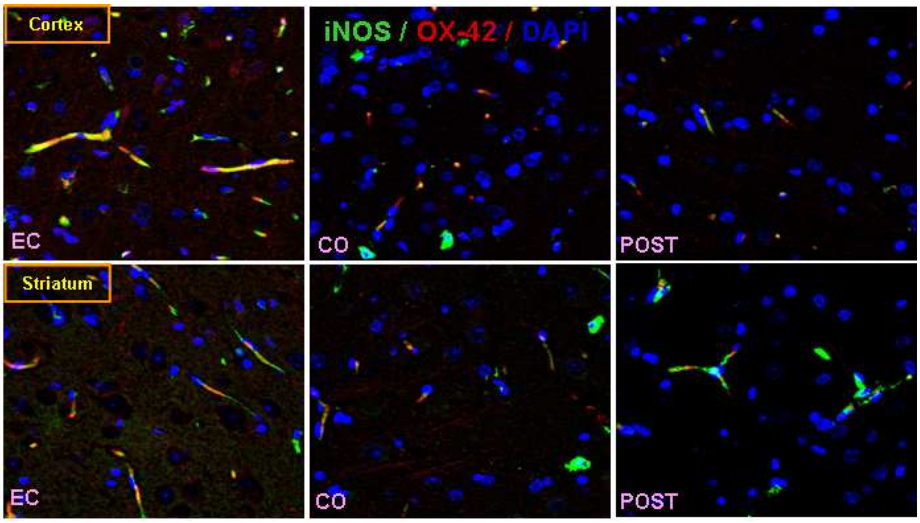
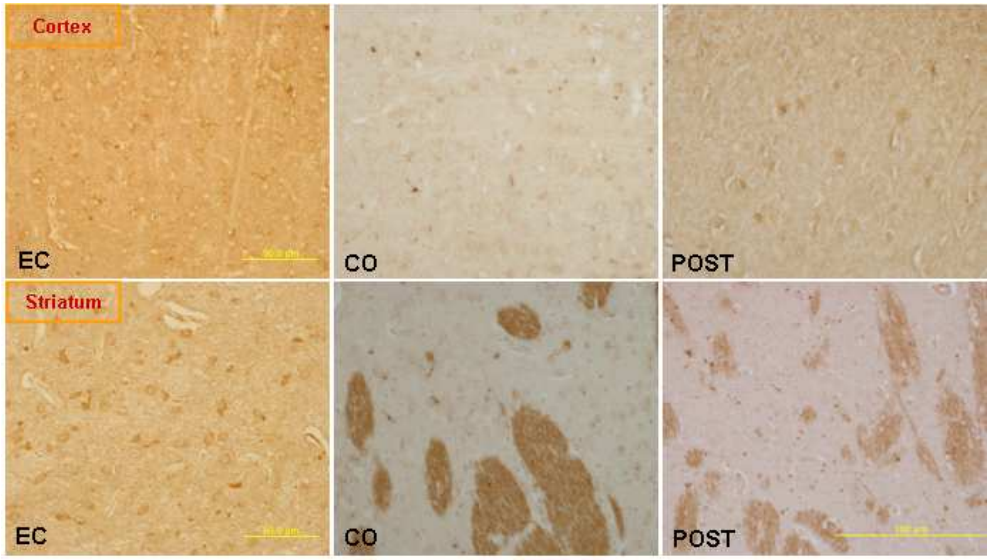


Fig 15. Identification of iNOS immunoreactive cells in the LPS stimulated brain (A). The number of iNOS positive cells was increased in EC group but, it was decreased both agmatine co and post-treatment group at 24 hours after LPS injected brain. Double labelling for OX-42 and iNOS (B). iNOS and OX-42 co-expressed in activated microglial cells in the cerebral cortex and striatum.

(A)



(B)

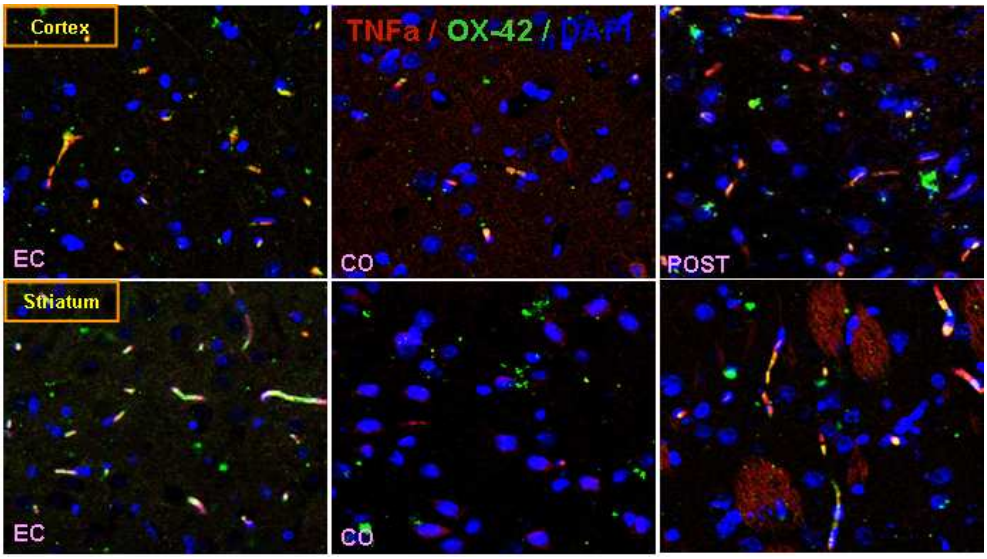
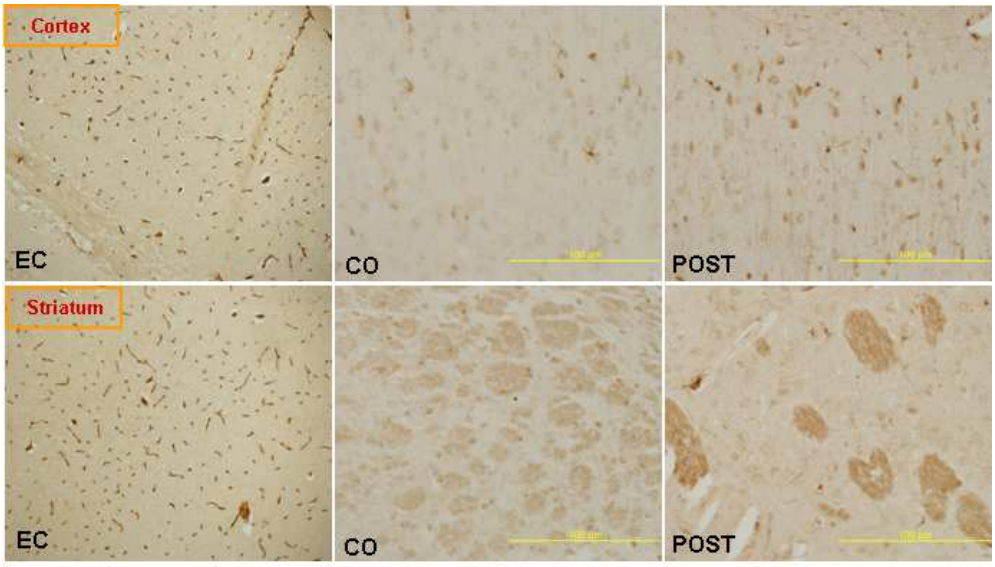


Fig 16. Micrograph of TNF-α immunoreactive cells in the LPS induced inflammatory injured brain. The number of OX-42, TNF-α positive cells was highly decreased both agmatine co-and post-treatment group at 24 hours after LPS injected brain (A). OX-42, TNF-α co-localized in activated microglial cells in EC group in the cerebral cortex and striatum (B).

(A)



(B)

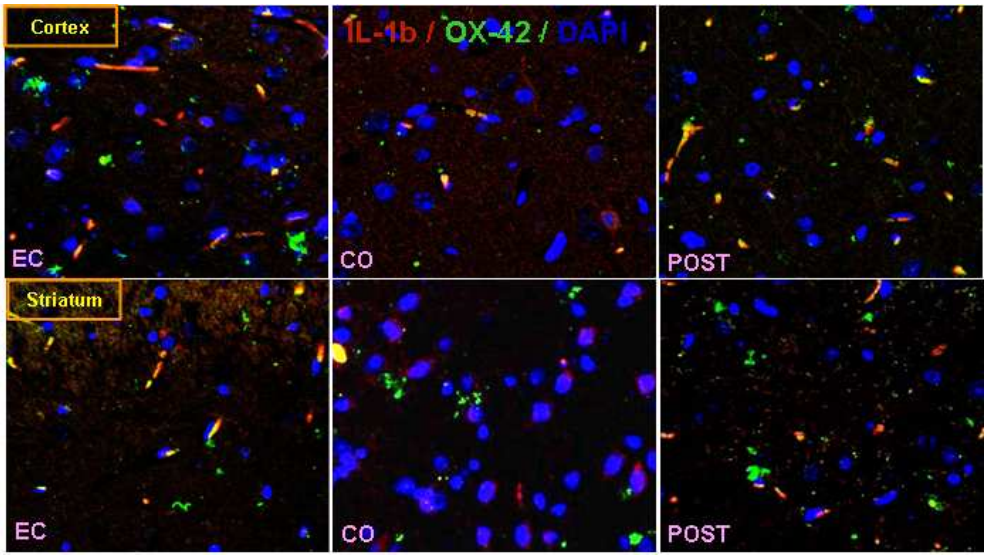
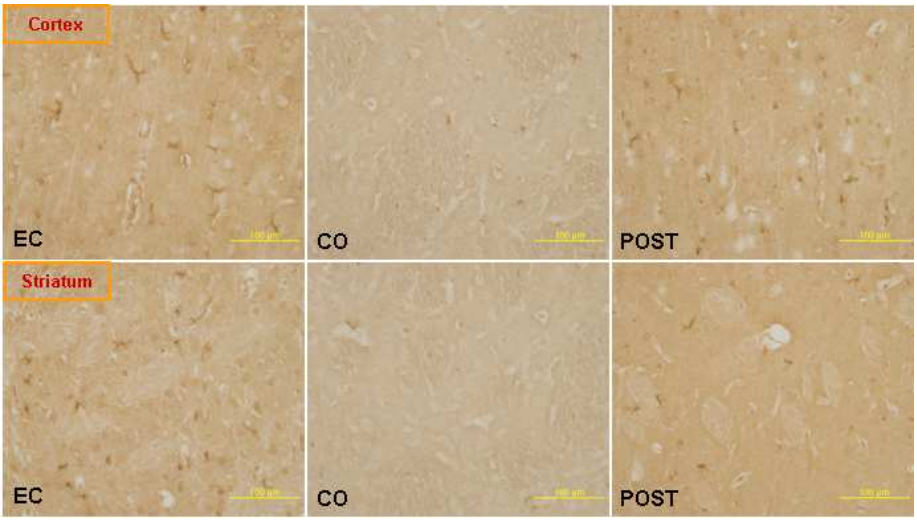


Fig 17. Micrograph of IL-1β immunoreactive cells in the LPS induced inflammatory injured brain. (A) OX-42, IL-1β co-localized in activated microglial cells in EC group in the cerebral cortex and striatum (B), but the number of OX-42, IL-1β positive cells was highly decreased both agmatine co and post treatment group at 24 hours after LPS injected brain.

(A)



(B)

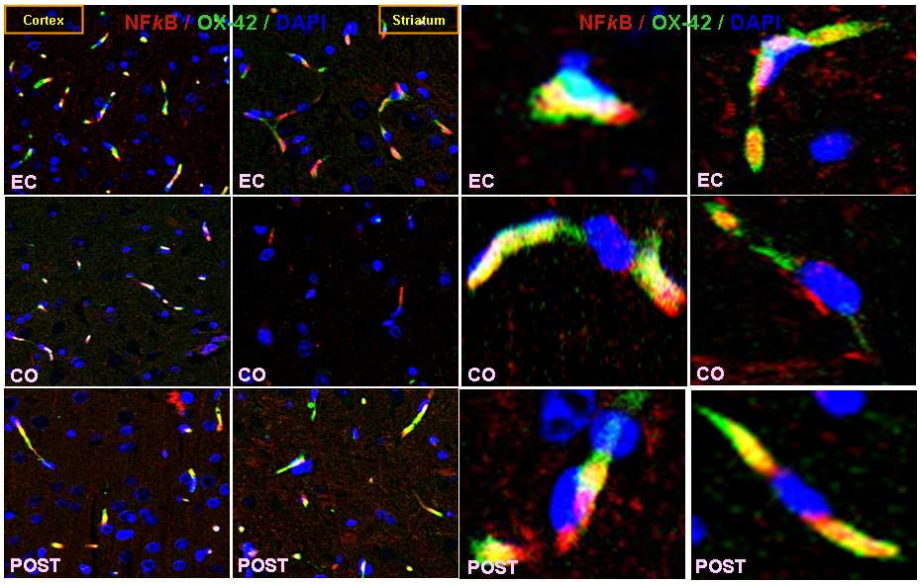
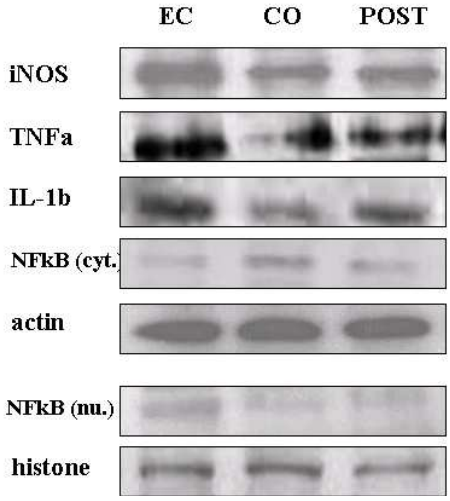


Fig 18. Micrograph of NF-κB immunoreactive cells in the LPS induced inflammatory injured brain. (A) OX-42 and NF-κB expressed in activated microglial cells in EC group in the cerebral cortex (A) and striatum (B), but the number of OX-42, NF-κB positive cells was highly decreased both agmatine co and post treatment group at 24 hours after LPS injected brain.

(A)



(B)

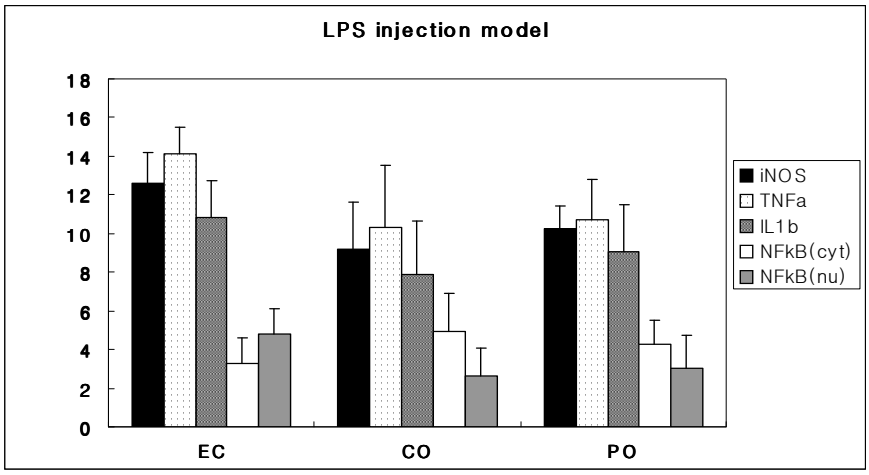


Fig 19. Western blot analysis of iNOS, TNF-α, IL-1β, and NF-κB in ipsilateral cerebral cortex at 24 hours after LPS intracerebral microinjection. 100 ㎍ of proteins were subjected to SDS-PAGE and transferred to PVDF membrane, and then blotted using antibody against NF-κB p65. Relative optical densities (ROD) of iNOS, TNF-α, IL-1β, and NF-κB are expressed as the band density of each group compared to the density of β actin. Agmatine co-treatment (CO) significantly decreased the expression of TNF-α iNOS, and IL-1β in cytoplasmic fraction and the expression of nuclear NF-κB also decreased compared to experimental control group (EC).

IV. DISCUSSION

Recent reports indicated that agmatine has neuroprotective effects against ischemic injury in neuronal cultures and experimental stroke *in vivo*[40]. Increased attention has been paid to brain injury associated with inflammatory processes. Focal cerebral ischemia triggers a pronounced inflammatory response involving activation of resident glial cells and recruitment of blood-derived leukocyte[39-41]. In this experiments, the effect of agmatine on neuroinflammation signaling in *in vitro* and *in vivo* ischemic and LPS induced injury was investigated. Agmatine treatment reduced cell death in microglial cells at 20 hours after OGD and LPS induced injury.

It has been reported that this protection by agmatine is associated with decreased NOS activity and expression, as well as NO generation[24,37]. NO is synthesized by three different NOS isoforms and is thought to function as both a neurotransmitter and a neurotoxin[42]. Microglia and astrocytes produce NO mainly from iNOS[43,44]. It has been reported that iNOS were induced in various types of central nervous injuries and diseases[45]. Also, iNOS expression in glial cells is triggered by LPS and certain cytokines[45-47] and the expression of iNOS has been identified in microglia cells in rodent brain after LPS treatment[8,48]. Microglia-derived NO has been presumed to be neurotoxic, therefore iNOS inhibitors[49] provided neuroprotective effects against LPS-induced neurotoxicity. Thus, glial derived high levels of NO has often been associated with pathological conditions[50]. Agmatine and nitric oxide are derived from the same substrate, arginine, and because these two products have opposite effects on inflammation, it was hypothesized that agmatine could suppress neuroinflammation through inhibiting NOS activity. In this study, the amounts of NO production was investigated from supernatants in cultured BV2 cells, which are microglial cell line. Agmatine reduced the production of nitrite and nitrate concentration. NO production by OGD or LPS stimulation was mainly due to iNOS induction as determined by Western blot analysis of iNOS protein in BV2 cells, as a result of that, the expression of iNOS protein was reduced by agmatine treatment.

Besides, a large number of iNOS immunopositive cells were identified in double-labelled cells with activated microglia marker proteins under inflammatory stimulated tissue, and agmatine decreased the number of iNOS positive cells in MCAO and LPS induced injury model. iNOS immunoreactivity was also expressed in microglial cells and astrocytes, and the number of iNOS positive cells were increased in ipsilateral cerebral cortex and striatum after 24 hours from MCAO injury respectively. The number of iNOS positive cells was also increased in ipsilateral cerebral cortex and striatum 24 hours after LPS induction. iNOS positive cells were overlapped OX-42 positive cells in LPS stimulated inflammatory brain.

Lipopolysaccharide (LPS) is a potent activator in inducing microglia activation in various neuron-glia cultures and subsequently causes neuronal cell death[51,52]. Activated microglia, are the major source of inflammatory cytokines in the CNS, and play a central role in brain inflammatory processes and this may be deleterious to neurons by production of toxic mediators like proinflammatory cytokines such as TNF-α, IL-1β, nitric oxide (NO), glutamate and free radicals[53]. Thus in the present study, LPS was used to investigate the alleviating effect of agmatine against microglial activation. These cytokines induce adhesion molecule expression, initiate thrombosis, and further activate other inflammatory cells[54]. So, blocking interleukin converting enzyme, which produces IL-1β, reduces injury in stroke animal model[55]. TNF-α is probably not present in normal brain tissue as evidenced by extremely low levels of TNF-α mRNA and peptide. However, in response to focal cerebral ischemia (transient or permanent), TNF-α mRNA can be detected in the brain as early as 1 hr post-ischemia[56], continues to signal strong transcription over 6-12 hrs and is still elevated for up to a week post-ischemia[57]. Like TNF-α, an increase in IL-1β mRNA expression has been shown to occur in cerebral ischemia and following lipopolysaccharide challenge. In addition, LPS is known to activate mitogen-activated protein kinases, nuclear factor-kappa B (NF-κB), protein kinase C and tyrosine kinases, which have been implicated in the release of immune-related cytotoxic factors, such as NO and proinflammatory cytokines[58-60]. As a result in this

study, agmatine treatment reduced the microglial activation in ischemic injured brain. The number of OX-42 positive cells were significantly decreased in agmatine treatment group after MCAO brain injury. And agmatine treatment also decreased the number of activated microglial cells after LPS treatment. The elevated level of TNF-α and that of IL-1β in BV2 cells with LPS stimulation were detected and in *in vivo* model, the expression of TNF-α and IL-1β were increased at protein level in MCAO injury or LPS microcerebral injection. In similar to iNOS immunohistochemical result, the number of TNF-α and IL-1β positive cells were increased in ipsilateral cerebral cortex and striatum after MCAO or LPS injury, respectively, and agmatine treatment decreased the number of each cytokine immunopositive cells significantly.

NF-κB is one of the most important transcription factors playing a pivotal role in mediating inflammatory responses to a variety of signals, including inflammatory cytokines, oxidative stress, hypoxia-reoxygenation. It has been investigated the NF-κB activation in microglial cells after MACO or LPS stimulated injury in this study. The number of NF-κB positive cells was increased in ipsilateral cerebral cortex and striatum 24 hours after MCAO or LPS injection. However, NF-κB positive cells reduced by agmatine treatment. On the other hand, the expression of nuclear NF-κB was increased in experimental control group, agmatine attenuated the expression of NF-κB in nuclear fraction at 24 hours after LPS. NF-κB is involved in acute phase of inflammatory responses which potentiate ischemic injury[18,61] by activating many genes involved in the pathogenesis of cerebral ischemia, such as iNOS, TNF-α, IL-1β, ICAM-1, COX-2[62]. Proinflammatory cytokines, such as TNF-α and IL-1β bind to their respective receptors and induce iNOS expression via activation of NF-κB[63-66]. The presence of multiple consensus sequences in the promoter region of iNOS for the binding of NF-κB and inhibition of NF-κB activation establish an essential role of NF-κB activation in the induction of iNOS[63-66]. In this study, it has been demonstrated that agmatine reduces microglial activation and decreased the production of proinflammatory cytokines and iNOS expression in the cerebral cortex and striatum at 22 hours reperfusion following MCAO injury and LPS

induction model, and experimental inflammatory *in vitro* model. These data indicated that agmatine suppressed the production of NO via inhibiting iNOS expression in microglia and reduced the expression of TNF-α and IL-1β through NF-κB translocation into nucleus during MCAO or LPS induced inflammatory injury. Thus, agmatine is suggested to have anti-inflammatory effect with suppression of microglial activation and NO production through regulation of NF-κB.

IV. CONCLUSION

Taken together, these data would be expected that agmatine reduces the production of NO and proinflammatory cytokines, such as TNF-α, IL-1β in *in vitro* and *in vivo* focal cerebral ischemia and inflammatory model. Agmatine may regulate the neuroinflammatory secretions and various proteins such as iNOS via regulation of NF-κB activation and exhibits anti-inflammatory properties in *in vitro* and in *in vivo* model of ischemic or inflammatory injury.

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국문초록

아그마틴에 의한 뇌허혈 후 동반되는 신경염증반응의 조절

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안 수 경

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염증반응은 중추신경계질환의 가장 큰 특징이며, 이는 아교세포의 활성화와 연관되어 있다. 미세아교세포는 중추신경계에 존재하는 면역반응세포로서, 뇌손상에 의해 활성화된 후 종양괴사인자-α (TNF-α), 인터루킨-1β (IL-1β)과 같은 전염증성 사이토카인과 일산화질소 (NO)를 포함하는 신경독성물질을 분비한다. 또한, 활성화된 아교세포는 염증반응과정의 중요한 구성요소가 된다.

아그마틴은 알기닌탈탄소효소에 의해 생성되는 필수아미노산이며, 아그마틴은 NOS의 길항제로서 일산화질소의 생성에 대한 내재성 조절체로 작용함이 보고된 바 있다. 생쥐에게 실험적인 뇌허혈 손상을 일으킨 후 아그마틴을 복강 내 주사하였을 때 신경보호작용에 대한 기전이 보고된 바 있다. 본 연구에서는 뇌허혈의 실험실모델인 중간대뇌동맥결찰술을 이용한 뇌허혈 동물모델과 LPS로 유도된 신경염증손상 모델에서 종양괴사인자-α,

인터루킨-1β와 같은 전염증성 사이토카인과 일산화질소의 생성에 미치는 아그마틴의 작용을 검토하였다. 대뇌허혈로 인해 생산되는 사이토카인의 분비에 대한 아그마틴의 역할을 규명하기 위해, BV2 미세아교세포주를 배양한 후 혐기성 챔버로 옮기고, 산소와 글루코스 결핍과 동시에 100 μM의 아그마틴을 처리하였다. 또한, 배양된 미세아교세포주를 1 ㎍/㎖ 의 lipopolysaccharide (LPS)로 염증반응을 일으킨 후, 100 μM의 아그마틴을 처리하였다. 그리고, 수컷 백서에 90분 동안 중간대뇌동맥결찰술을 시행함과 동시에 100 ㎎/㎏의 아그마틴을 복강 내 주사하거나, 재관류가 시작될 때 동량의 아그마틴을 주사하였다.

아그마틴은 산소와 글루코스결핍에 의한 미세아교세포의 손상과 LPS로 유도된 염증성 손상시 세포사멸을 감소시켰다. 이러한 세포보호효과는 아그마틴이 NOS의 활성, 발현 뿐 아니라 일산화질소의 생성과 연관되어 있다고 보고된 바 있으므로, 본 연구에서도 뇌허혈 손상과 신경염증 손상 후 아그마틴에 의한 신경보호작용이 NO의 생성과 이를 생성하는 iNOS 효소의 발현과 어떠한 연관성이 있는 지 조사하였다. 본 연구에서 중간대뇌동맥결찰 후 동측 대뇌피질과 선조체에서, inducible nitric oxide synthase (iNOS) 양성반응세포의 수가 증가하였고, iNOS 면역양성반응은 미세아교세포 뿐 아니라 별아교세포에서도 확인되었다. 그리고, 중간대뇌동맥결찰술과 LPS로 유도된 염증성 손상 시 발현되는 iNOS 양성반응세포의 수가 아그마틴에 의해 감소함을 확인하였다. 또한, 종양괴사인자-α, 인터루킨-1β와 같은 전염증성 사이토카인과 iNOS, nuclear factor-ĸB (NF-ĸB)항체를 이용하여 면역조직화학과 Western blot 분석을 시행하였다. 중간대뇌동맥결찰술과 LPS로 유도된 염증성 손상 후 동측 대뇌 피질과 선조체에서 종양괴사인자-α와, 인터루킨-1β 면역양성반응세포의 수가 각각 증가하였으며, 아그마틴에 의해 그 수가 유의하게 감소하였고, NF-κB 면역양성반응세포 수 또한 아그마틴에 의해 감소하였다. 아그마틴은 미세아교세포의 활성을 억제시키며, 아그마틴을 주사한 군에서는 미세아교세포에서 종양괴사인자-α와, 인터루킨-1β, iNOS, NF-ĸB 항체에 대한 면역반응활성이 유의하게 감소하였으나 활성화된 별아교세포 수의 변화에는 유의성이 없었다.

이상과 같은 결과는 중간대뇌동맥결찰술이나 LPS로 유도된 염증성 손

상 후 미세아교세포에서 아그마틴이 iNOS의 발현을 억제함으로써 일산화질소의 생성을 감소시키고, 핵 안으로의 NF-κB translocation을 감소시킴으로써 종양괴사인자-α, 인터루킨-1β의 발현이 감소함을 나타낸다. 이상의 결과로부터 아그마틴이 미세아교세포의 활성과 NF-κB 조절을 통해 일산화질소의 생성을 억제하는 항염증효과가 있을 것으로 생각된다.

핵심되는 말 : 대뇌허혈, 염증, LPS, 아그마틴, iNOS, 사이토카인