The effect of agmatine on murine cortical endothelial cells under ischemic injury

Thesis by

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The effect of agmatine on murine cortical endothelial cells under ischemic injury

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This certifies that the Doctoral Dissertation of

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이국에서의 지난 3 년간, 많은 분들의 따뜻한 관심과 도움을 받으면서 또 많은 것을 느끼면서 제 인생에서 소중한 기억으로 남을 시간이었습니다.

제가 여기까지 올 수 있도록 가르쳐주시고 이끌어주신 지도교수님이신 이종은 교수님께 진심으로 감사를 드립니다. 보이지 않게 여러 가지로 많은 도움을 주신 박경아 교수님, 이원택 교수님 감사합니다. 본 논문을 심사하시면서 조언을 아끼시지 않으신 약리학 교실의 안영수 교수님, 신경과학 교실의 허지회 교수님께도 고마움을 전합니다. 3 년 동안 같이 생활해온 실험실 모든 분들께 감사의 마음을 전하고 싶습니다. 신영호 선생님, 정효석 선생님, 안수경, 김재환, 김종열, 윤성환, 문진희, 김한조, 김사현, 이용우, 김지희, 그리고 최윤정 감사합니다.

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ABSTRACT

The effect of agmatine on murine cortical endothelial cells under ischemic injury

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Agmatine is formed by the decarboxylation of L-arginine by the arginine decarboxylase (ADC). In this study, we found that agmatine decreased the MMPs expression in mouse cerebral endothelial cells. Matrix metalloproteinases (MMPs) are up−regulated by ischemia and degrade the basement membrane of brain vesssels to promote cell death and tissue injury. The *e*NOS expressed in the endothelial cells and nitric oxide generated by this enzyme play an important role in regulation of vascular reactivity. In this study we investigated the effect of agmatine administered exogenously and endogenously through overexpression of ADC. We performed RT-PCR and western blot analysis against MMP-2, MMP-9 and *e*NOS, and measured the production of NO using Griess reagent. While the expression of *e*NOS was

increased after ischemic injury, the expression of MMPs was decreased by agmatine administered exogenously and endogenously. We also showed L-NAME (NOS inhibitor) altered the suppression of MMP-9 by exogenously administered agmatine, but not that of MMP-2. It seems that MMP-9 suppression by exogenously administrated agmatine is mediated, at least in part, via *e*NOS and the maintenance of functional NO release. Furthermore, we thought transcriptional regulation of MMP gene expression is influenced by some transcription factors. Activating transcription factor 3 (ATF_3) is rapidly induced in response to a variety of stress such as ischemia reperfusion injury in endothelial cells. We found that ATF_3 expression was increased significantly in ADC overexpression cells, but it was attenuated by NOS inhibitor. It seems that ATF_3 expression is mediated *e*NOS in ADC overexpression cells. Furthermore, we found that the suppression of MMP-2 and MMP-9 by agmatine were attenuated in cells transfected with ATF_3 siRNA. Our study indicated that the inhibition of MMPs expression by endogenously agmatine might be mediated via the regulation of ATF_3. Taken together, these results suggest that endogenously administered agmatine suppress the MMP-2 and MMP-9 expression via *e*NOS-NO-ATF_3-MMPs pathway.

Key words: agmatine, arginine decarboxylase, matrix metalloproteinase, endothelial nitric oxide synthases, nitric oxide, activating transcription factor 3

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Ⅰ. Introduction

Agmatine, a polycationic amine synthesized by decarboxylation of L-arginine by the arginine decarboxylase (ADC), was first discovered in 1910. It has been shown to exert some neuromodulatory functions in the central nervous system[1, 2] and have neuroprotective effect in trauma and neonatal ischemia models[3, 4, 5, 6, 7]. Recently, agmatine, ADC, and agmatinase were found in mammalian brain[1, 3] and agmatine appears to act on endothelial cells to increase the synthesis of nitrite and nitric oxide, a vasodilatory substance [8]. Agmatine and L-arginine produced a decrease in systemic blood pressure in

rats and rabbits and this decrease was sensitive to the NOS inhibitor, L-NAME[9]. Agmatine also exerted protective effects against ischemia-reperfusion injury in the isolated heart and the recovery was attributed to its vasodilatory effects[10, 11, 12]. With the effect by agmatine, it is indeed interesting to study the relationship of agmatine with NO and vascular endothelial cells.

The extracellular matrix molecules constitute the basement membrane underlying the vasculature and play a critical role for providing structural support to the endothelial cell wall. Matrix metalloproteinases (MMPs) are a group of zinc-dependent enzymes that may contribute to the pathophysiology of cerebral ischemia by degrading the matrix components in the neurovascular unit. An early and major pathological effect of MMPs in cerebral ischemia is the disruption of the blood-brain barrier (BBB) through the degradation of basal lamina that surround the cerebral blood vessels. Members of the MMP family, MMP-2 and MMP-9 are well-known, which degrade the extracellular matrix components of the basement membrane of brain vessels[13, 14, 15].

The vascular endothelium mediates the ability of blood vessels to alter their architecture in response to hemodynamic changes. However, the specific endothelial-derived factors that are responsible for vascular remodeling are poorly understood. Here we show that endothelial derived nitric oxide is a

major endothelial-derived mediator controlling vascular remodeling. Nitric oxide is a multifunctional messenger molecule generated from L-arginine by the family of isoenzymes called nitric oxide synthases (NOS)[16, 17]. The *e*NOS expressed in the endothelial cells and Nitric oxide generated by this enzyme plays an important role in the regulation of vascular reactivity[16, 18]. The production of NO by *e*NOS plays a protective role in cerebral ischemia[19]. *e*NOS knock out mice exhibit an increase in vessel wall thickness and NO derived from *e*NOS is a major regulator of vessel reorganization in response to a remodeling stimulus[20]. A non-enzymatic NO release was also observed in the vascular system, to affect the vascular tone during ischemia-reperfusion injury[18]. So, agents that enhance NO formation have a great therapeutic potential for ischemia-reperfusion injury.

Ischemia– reperfusion injury induces endothelial cytotoxicity and a blood vessel injury induces extracellular matrix turnover. Nitric oxide has an important influence on MMP expression in the vascular tissue and may mediate the vessel wall remodeling by regulating the expression of matrix metalloproteinases[14, 21]. While the induction of MMP and *e*NOS are two separate processes, the interaction between these two molecules has been suggested. When *in vitro* aortic tissue was stimulated in the presence of NOS

inhibitor, there was an increase in the MMP-9 protein level and mRNA expression[21].

Activating transcription factor 3 (ATF_3) is rapidly induced in response to a variety of stress such as ischemia reperfusion injury in endothelial cells[22, 23], suggesting that it plays a role in the stress-responsive pathway. Kawauchi et al. clearly demonstrated that ATF_3 acts as a transcriptional repressor to protects endothelial cells from tumor necrosis factor-induced apoptosis[24]. It was shown that NO induced a dose and time dependent-induction of ATF_3 expression and the ATF_3 induction resulted in the inhibition of MMP-2 promoter activity[25].

The present study was aimed to determine whether exogenously and endogenously administered agmatine decrease the MMP-2 and MMP-9 expression after oxygen-glucose deprivation (OGD) and reperfusion injury in cerebral endothelial cells (CECs), and to evaluate whether the effect is related to the *e*NOS. We also investigated the role of ATF_3 in the inhibitory effect of NO on MMP−2 and MMP−9 expression.

Ⅱ. Materials and Methods

1. Materials

M199 and DMEM were purchased from GIBCO. The anti MMP-2 and MMP-9 antibodies were purchased from Calbiochem (Chemicon, CA, USA), anti *e*NOS antibody was purchased from Transduction Lab. (BD Transduction Lab. Hercules, CA, USA). Anti-ATF_3 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence (ECL) western blotting detection system were obtained from Amersham Biosciences (ECL Plus, Amersham International PLC, Little Chalfont, UK). The inhibitors were obtained from Sigma (St. Louis, MO, USA). Other enzymes and chemicals were from Sigma. Fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG and Texas Red-conjugated goat anti-mouse IgG were from Molecular Probes. DAPI (49,6-diamidino-2-phenylindole dihydrochloride) was from nacalai tesque (Kyoto, Japan).

2. Primary murine brain endothelial cell culture

Primary mouse brain endothelial cells were cultured from brain microvessels isolated from the cortical grey matter of mouse. Briefly, 20 two

weeks old mouse were anesthetized with Ether and heads were washed by dipping in 70% ethanol for 10 sec then decapitated. Cortices in HBSS (Hank's balanced salt solution) were prepared, cortical hemispheres were taken out and inserted into the HBSS and white matter and meninges were removed. The cortices in the incubation solution Ⅰ (0.35% collagenase in M199) were incubated at 37℃ for 2 h then washed in 2.5% BSA. The pellet was incubated in solution Ⅱ (0.1% collagenase) again at 37℃ for 2 h then dissolved in a 2 ml M199 medium. The capillary fragments of this suspension were separated by centrifuging through a Percoll gradient. The layer containing the capillaries was removed from the gradient, washed and suspended in M199 culture medium containing 10% fetal bovine serum, endothelial cell growth supplement (0.75 μg/ml), heparin (80 μg/ml), ascorbic acid (5 μg/ml), L-glutamine (2 mM) and antibiotics. Endothelial cells from this preparation were grown in culture flasks pre-coated with collagen. Confluent endothelial cells were replated by trypsinisation and used in experiments at passage 2[26, 27, 28].

3. bEnd.3 cell line culture.

For protein and mRNA assay, bEnd.3 cells were used. The bEnd.3 cells were cultured in a media containing of Dulbecco's modified Eagle's medium

(GIBCO) supplemented with 4 mM L-glutamine, 4500 mg/L glucose, 1 mM sodium pyruvate, 1500 mg/L sodium bicarbonate, penicillin (500 u/ml), streptomycin (100 μg/ml) and 10% fetal bovine serum (FBS).

4. ADC infection

For this study, we constructed the retroviral vector containing human ADC cDNA and established a transfection of human ADC gene into the murine bEnd.3 cells (bEnd.3-ADC) by the retroviral packaging cell line PT67[29].

5. Oxygen-glucose deprivation (OGD)

Confluent cells were transferred into a temperature controlled (37±1℃) anaerobic chamber (Forma Scientific, OH, USA) (O_2 tension < 0.1%), washed three times with deoxygenated, glucose-free balanced salt solution (BSS_0) containing 116 mM NaCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 5.4 mM KCL, 1 mM NaH_2PO_4, 14.7 mM $NaHCO_3$, 10 mM HEPES, 10 mg/L phenol red at pH7.4. And cells were maintained in the hypoxia chamber for 6 h and returned to the incubator adding glucose to the culture medium to a final concentration of 5.5 mM for 18h under 5% CO2/95% air condition. Normal control (NC) cells were not exposed to OGD. Agmatine (100 μM) was added to the culture medium 30 min before OGD (PRE treatment group, pre), at the

start of injury (CO treatment group, co), and at reperfusion when glucose and oxygen were restored (POST treatment group, post)[30, 31].

6. LDH Assay

Mouse cerebral endothelial cell death was also quantitatively assessed by measuring the extent of LDH release into the medium after OGD for 6 h and normoxic 16 h. The amount of total LDH released of 100% cell death named "full kill" was determined at the end of each experiment following freezing at -70℃ and rapid thawing. The extent of cell death was expressed as percentage of full kill.

7. Immunocytochemical analysis

Primary cultured mouse cerebral endothelial cells were plated onto collagen coated 24-well plate and fixed with 4% paraformaldehyde for 15 min, and permeabilized with 0.5% Triton X-100 for 10 min. Blocking was performed for 3vh at room temperature in blocking solution (zymed. bulk) for 3 h. Cells were incubated at room temperature overnight with a primary antibody rabbit anti-MMP-2 (1:500) and rabbit anti-MMP-9 (1:500). Cells were then treated with a conjugated secondary antibody (zymed. bulk) for 45 min. Enzyme conjugated reagent (zymed. bulk) was added for 20 min before

the DAB reaction (sigma D-4293) for 2-10 min and washed with PBS[33]. For counter-staining of nuclei, bEnd.3 cells and bEnd.3ΔADC cells were plated in a chamber slide. And immunostained as described above, then cells were treated with DAPI. Then observation in an Olympus microscope equipped for epifluorescence with UV.

8. Measurement of NO

To assess NO production, the measurement of the stable end-productions of NO metabolism, nitrite (NO^{2-}) and nitrate (NO^{3-}), were used based on the Griess reaction. 100 μL of supernatants were mixed with 100 μL of Griess reagent. The absorbance at 540 nm with a reference wavelength at 595 nm was measured, and nitrite concentration was determined using a calibration curve with sodium nitrite standards[34, 35].

9. MCAO model

Male ICR mice weighing 38-40 g were subjected to transient middle cerebral artery occlusion (MCAO). Animal were anesthetized with 4% chloralhydrate, i.p. Rectal temperature, respirations, heart rate were monitored and maintained in the physiologic range throughout the surgery. Ischemia was induced using an occluding intra luminal suture. In brief, a cervical midline

incision was made and the left carotid artery and branches were isolated. An uncoated 15 mm long segment of 6-0 Dermalon suture (blue monofilament nylon DG, Ethicon, Somerville, NJ) with the tip rounded by a flame was inserted into the arteriotomy and advanced under direct visualization into the internal carotid artery (ICA) approximately 11 mm from the bifurcation in order to occlude the ostium of the MCA. At the end of the ischemic period, the suture was removed and surgical incisions were closed and the animals were allowed to recover. Twenty-two hours later, animals were sacrificed. Agmatine (100 mg/kg of mouse, IP) were injected following the scheme[32].

10. Immunohistochemical analysis

Mice were sacrificed, and the brains were quickly removed and fixed with 10% phosphate-buffered formalin for 18-20 h. After standard processing and embedding in paraffin, 6-um-thick sections were prepared. Endogenous peroxidase was quenched with 0.3% H_2O_2 in methanol for 30 min. Nonspecific immuoglobulin binding sites were blocked with blocking solution, and then the sections were incubated with a primary antibody for 1 hour, secondary antibody for 30 min at room temperature. Immunoglobulin complexes were visualized on incubation with DAB (Zymed Laboratories) enhancing solution and then examined by light microscopy with microscope.

The primary antibodies used for these studies were s follows: anti-MMP_2 (1:500), mouse anti-MMP_9 (1:500), CD_{31} (1:1000)[36].

11. Western blot analysis

Proteins were isolated from endothelial cells and lysed in solubilizing buffer (1×PBS, 1% nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, protease inhibitors-PMSF, aprotinin and sodium orthovandate). Equal amounts of protein extracts were separated by 10% SDS-PAGE and transferred to a PVDF membrane. The membrane was blocked with 5% nonfat milk in TBS containing 0.05% Tween 20 and reacted with antibody rabbit anti-MMP-2 (1:2000), rabbit anti-MMP-9 (1:2000) and rabbit anti-eNOS (1:1500). The membrane was then incubated with the secondary antibody (1:2000, anti rabbit) and thoroughly washed. Immunoreactive bands were visualized with an ECL (ECL Plus, Amersham international plc, Little Chalfont, UK) and auto-radiographed[37].

12. RT- PCR Reaction

Total RNA was isolated and purified with Trizol Reagent (Invitrogen, Carlsbad, CA, USA) according to the protocol recommended by the manufacturer. RNA was quantitated by measuring the absorbance at 260 nm,

and the ratio was 1.8 or higher. cDNA synthesis of mRNA was carried out by reverse transcription (RT). Normalization of the samples was accomplished using the reverse transcriptase-polymerase chain reaction (RT-PCR). PCR amplification for MMP-2 was performed at 94℃ for 30 sec, at 53℃ for 30 sec and at 72℃ for 30 sec for 35 cycles and the PCR amplification for MMP-9 was performed at 94℃ for 30 sec, at 59℃ for 30 sec and at 72℃ for 30 sec for 30 cycles. The sequences of the specific primers were as follows: sense, 5'- GAG TTG GCA GTG CAA TAC CT-3', and antisense, 5'- GCC GTC CTT CTC AAA GTT GT-3' for MMP-2; sense, 5'- TTA CCA GCG CCA GCC GAC TTT TG- 3' and antisense, 5' –CGT CGT CGT CGA AAT GGG CAT C-3' for MMP-9; sense, 5'-TTCAACACCCCAGCCATGT-3' and antisense, 5'-TGTGGTACGACCA GAGGCATAC-3' for ß-actin. The PCR products were separated by electrophoresis in 1.5% agarose gels with ethidium bromide[38, 39].

13. siRNA construction

bEnd.3ΔADC cells were transfected with small interfering RNA (siRNA) designed to suppress ATF_3 expression. Following the manufacturer's instructions, bEnd.3ΔADC cells were plated onto 12-well plate for protein preparation and they were transfected at 70% confluency with 10 nM ATF_3

siRNA using the Lipofectamine reagent (invitrogen, Carlsbad, CA, USA). After transfection, cells were recovered in the regular growth medium for 48 h. Two days after transfection, cells reached confluence, and were exposed to OGD reperfusion injury. ATF_3 siRNA effect was measured by western blotting.

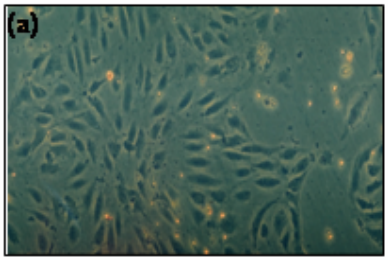
14. Statistical analysis

Results represent means ±S.E.M. Statistical analyses were performed using the GraphPad Instat software (GraphPad Software, Inc). All the data were analyzed using the Student's t-test or ANOVA. Differences were considered significant at $p< 0.05$.

Ⅲ. Results

1. Identifying the presence of primary cultured endothelial cells

Immediately after seeding, cerebral endothelial cells from murine brains, associated with cell clusters, were attached to the collagen-coated surfaces within 24-36 h. After 3-4 days in culture, the cells reached confluence post-seeding. With increasing cultivation time, cell clusters disappeared and the morphologically homogeneous growth could be observed. The typical morphology of a confluent endothelial cell monolayer is shown in Fig.1(a). Endothelial cells were characterized by the presence of von Willebrand factor (vWF, endothelial cell marker). To detect the presence of factor-Ⅷ antigen in endothelial cells, we performed immunocytochemistry. The primary cultured mouse capillary endothelial cells were detected by the positive staining of vWF as shown in Fig.1(b).



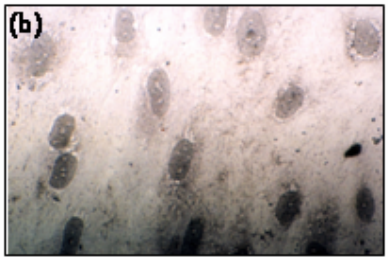


Fig. 1. Identification of the primary cultured endothelial cells. Primary murine brain endothelial cells were cultured from brain microvessels isolated from the cortical grey matter of mouse. Endothelial cells from this preparation were grown in culture flasks pre-coated with collagen. Figure shows the phase-contrast micrograph of cerebral capillary endothelial cells at culture day 4 (a). Cerebral capillary endothelial cells are positively stained with Factor Ⅷ (b).

2. Immunocytochemical analysis of MMP-2 and MMP-9 for primary cultured murine cortical endothelial cells after OGD

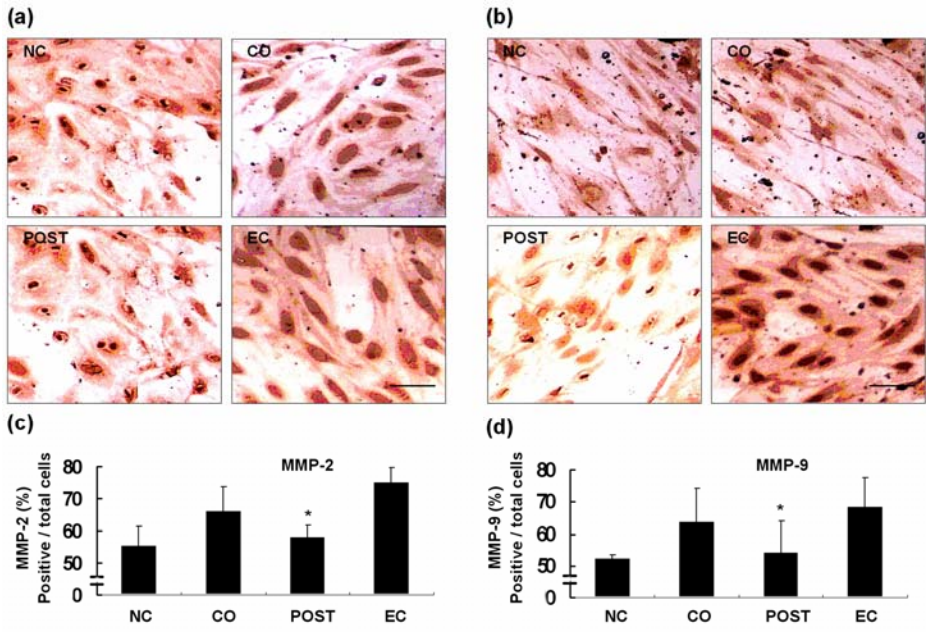


Fig. 2. Immunocytochemical analysis of MMP-2 and MMP-9 in primary cultured murine cortical endothelial cells. The primary murine brain endothelial cells were grown in culture flasks pre-coated with collagen. Confluent endothelial cells were replated by trypsinisation and used in experiments at passage 2. And cells were maintained in the hypoxia chamber for 6 h and returned to the incubator, adding

glucose to the culture medium to a final concentration of 5.5 mM for 18 h under 5% CO_2/95% air condition. Normal control (NC) cells were not exposed to OGD. Agmatine (100 μM) was added to the culture medium at the start of injury (CO treatment group, co), and at reperfusion when glucose and oxygen were restored (POST treatment group, po). Data is expressed as the percentage of immuno-positive cells to the total cell number. Data represent means ± SEM (N=6). *P<0.05.

As shown in Fig. 2, agmatine led to the reduction of MMP-2 and MMP-9 in primary cultured cerebral endothelial cells. The MMP-2 and MMP-9 immuno-positive cells were significantly reduced in the post-agmatine treatment group compared to the experimental control (EC).

3. Measurements of NO

The concentration of nitrite in the culture medium was shown similarly in the primary cultured endothelial cells (Fig.3) and the mouse brain endothelial cell line (Fig.4). It was shown that OGD-reperfusion injury decreased the concentration of NO production in endothelial cells. However, the agmatine treated-groups demonstrated an attenuated decrease of NO compared to EC group, especially in the post-agmatine treatment group. . As shown in Fig.3B, a further experiment was carried out to examine the possible additive effect of NO production by NOS inhibitor N (G)-nitro-L-arginine (L-NAME)[40]. Co-

administration of L-NAME and agmatine did not alter NO production significantly, as shown in Fig. 4. These observations suggest that the agmatine effect on NO reduction is related to NOS.

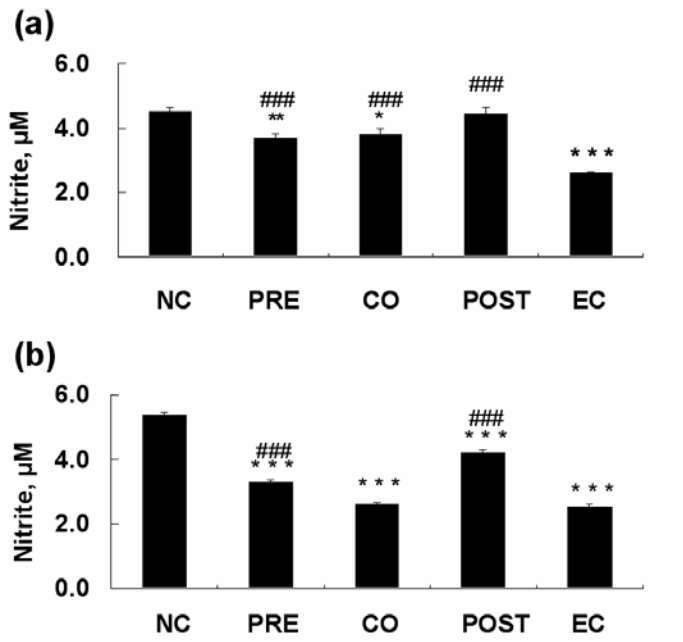


Fig. 3. Effect of agmatine on NO production in primary murine cortical endothelial cells and bEnd.3 mouse brain endothelial cell line. Agmatine influence on the NO production after OGD in primary mouse brain endothelial cells (a) and bEnd.3 mouse brain endothelial cell line (b). Agmatine (100 μM) was added to the culture medium 30min before OGD (PRE treatment group, pre), at the start of injury (CO treatment group, co), and at reperfusion when glucose and oxygen were restored (POST treatment group, post). Data represent means ± SEM (N=6). * P<0.05, * * *P<0.01, * * * P<0.001; denotes significant difference compared with normal control. ### denotes significant difference from the experimental control.

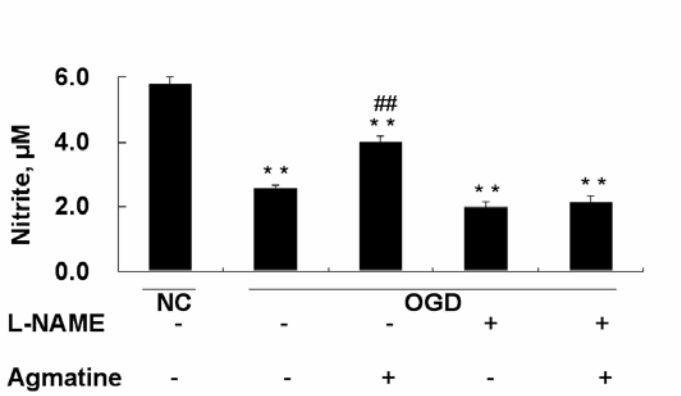


Fig. 4. Effect of co-administration of L-NAME and agmatine on NO production following OGD in bEnd.3 mouse brain endothelial cells. Agmatine and L-NAME were added to the culture medium at the start of reperfusion. Data represent means ± SEM (N=6). * * P<0.01; denotes significant difference compared with control. ## denotes significant difference from the none agmatine or (and) the L-NAME treated experimental control.

4. Agmatine modulate the protein expression of *e*NOS, MMP-2 and MMP-9

To determine whether the expression of *e*NOS protein levels correlated with agmatine, western blotting was conducted in normal control (NC), experimental control (EC) and agmatine treated-groups. The expression of *e*NOS was decreased after OGD reperfusion injury. Agmatine prevented the reduction of *e*NOS during OGD and reperfusion injury in bEnd.3 cells.

However, the expression of MMPs was decreased by agmatine after ischemic injury, especially on MMP-9. And the expression of MMP-2 was decreased only in the post agmatine treated group (Fig. 5.).

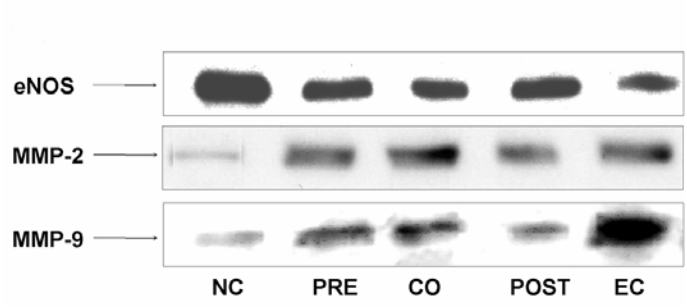


Fig. 5. Agmatine modulate the protein expression of *e*NOS, MMP-2 and MMP-9. Total protein samples were collected and analyzed for *e*NOS, MMP-2 and MMP-9 by western blotting in bEnd.3 cells. Agmatine (100 μM) was added to the culture medium 30 min before OGD (pre), at the start of injury (co), and at reperfusion when glucose and oxygen were restored (post).

5. The effect of co-administration of L-NAME and agmatine on MMP-2 , MMP-9 and *e*NOS protein expression

To investigate whether the expression of MMPs is related to the *e*NOS, we exert a further inhibitory study. The expression of *e*NOS was suppressed by L-NAME. And, we found that the suppression of MMP-9 by agmatine was attenuated by L-NAME which is an inhibitor of NOS. As shown in Fig. 6, the

combination of L-NAME with agmatine decreased MMP-2 expression, but did not suppress MMP-9. Therefore, L-NAME altered the effect of agmatine on MMP-9 suppression, but not MMP-2.

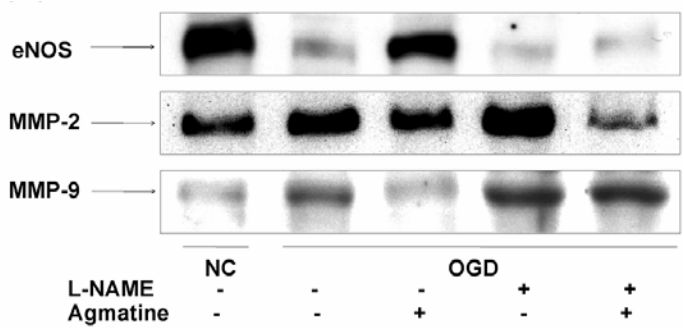


Fig. 6. Effect of co-administration of L-NAME and agmatine on MMP-2 , MMP-9 and *e*NOS protein expression. Total protein samples were collected and analyzed for *e*NOS, MMP-2 and MMP-9 by western blotting in bEnd.3 cells. Results show the effect of combined administration of agmatine and L-NAME on *e*NOS, MMP-2 and MMP-9 protein expression in bEnd.3 cells after OGD reperfusion injury. Agmatine and L-NAME were added to the culture medium at the start of reperfusion.

6. Effect of agmatine on mRNA levels of MMP-2 and MMP-9

Similarly, RT-PCR analysis demonstrated that agmatine also modulates MMP-2 and MMP-9 expression at the mRNA level. Co-administration of L-NAME and agmatine decreased MMP-2 mRNA while MMP-9 mRNA level was not altered. In this regard, agmatine regulates MMP-2 and MMP-9

expression at the mRNA levels and NOS inhibition is associated with the MMP-9 mRNA expression.

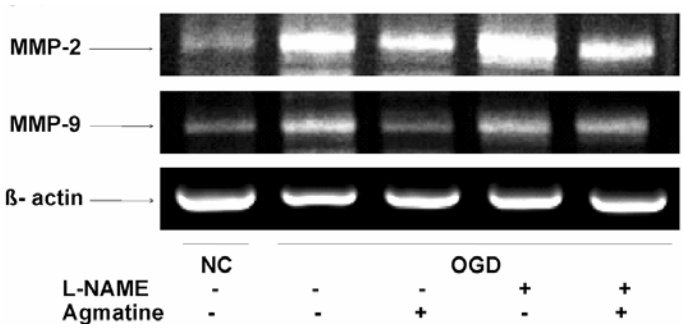


Fig. 7. Effect of agmatine on mRNA levels of MMP-2 and MMP-9. The data shows the effect of the combination of L-NAME with agmatine treatment on levels of mRNA for MMP-2 and MMP-9. Agmatine and L-NAME were added to the culture medium at the start of reperfusion. Total RNA was isolated and the RT-PCR ethidium bromide-stained gels showed the expression of MMP-2 and MMP-9.

7. Retroviral- ADC infection and identification

We constructed the retroviral vector containing human ADC cDNA and established a transfection of human ADC gene into the murine bEnd.3 cells by the retroviral packaging cell line PT67. The cell line transfected with a stably high expression of ADC was identified in RNA level.

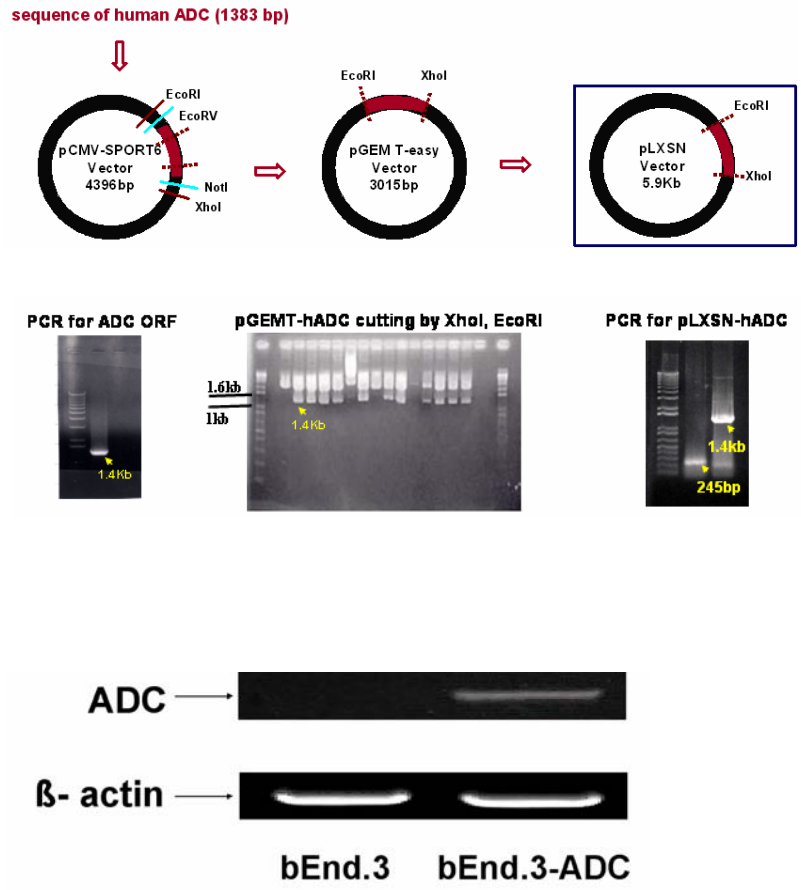


Fig. 8. Retroviral- ADC infection and identification. Manufacture of vector for retroviral hADC expression. The bEnd.3 cells demonstrate no detectable ADC mRNA expression, as compared with bEnd.3-ADC cells.

8. Effect of agmatine administered exogenously and endogenously on LDH and NO production

We carried out assay of lactate dehydrogenase (LDH) release. It was shown

in Fig. 9. After OGD-reperfusion injury, LDH level in the medium was significantly increased. However, bEnd.3 cells with overexpressed ADC attenuated LDH release significantly under OGD-reperfusion injury compare to EC group (P<0.01).

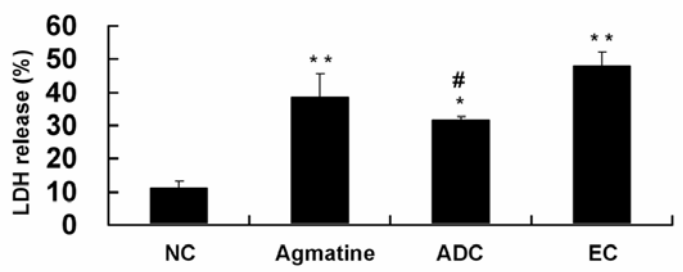


Fig. 9. Effect of agmatine administrated exogenously and endogenously on LDH release. bEnd.3 cells were exposed to normoxia (NC) or OGD-reperfusion injury (Agmatine, agmatine was added at the start of reperfusion; ADC, ADC overexpression cell; EC, experiment control). The results are presented as a percentage value. Data represent means ± S.E.M (N=6). * P<0.05; * * P<0.01; denotes significant difference compared with normal control (NC). ## denotes significant difference from the experimental control.

Ischemia-reperfusion injury decreased the concentration of NO production in endothelial cells. However, the agmatine administered exogenously and endogenously attenuated the decrease of NO compared to the EC, especially in the ADC overexpressioned cells (P<0.01) (Fig. 10).

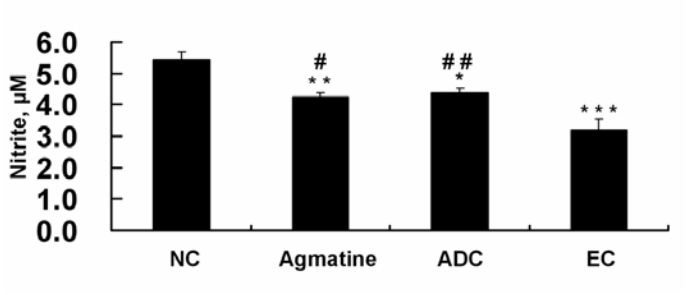


Fig. 10. Agmatine administered exogenously and endogenously influence on the NO production. bEnd.3 cells were exposed to normoxia (NC) or OGD-reperfusion injury (Agmatine, agmatine was added at the start of reperfusion; ADC, ADC overexpression cell; EC, experiment control). Data represent means ± S.E.M (N=6). * P<0.05; * * P<0.01; denotes significant difference compared with normal control (NC). ## denotes significant difference from experimental control (EC).

9. Effect of agmatine administered exogenously and endogenously on MMPs, *e*NOS and ATF_3 regulation

The expression of *e*NOS was decreased after OGD reperfusion injury. Agmatine administered exogenously and endogenously prevented the expression of *e*NOS during OGD and reperfusion injury. However, the expression of MMPs was decreased, especially in agmatine exogenously and endogenously administered.

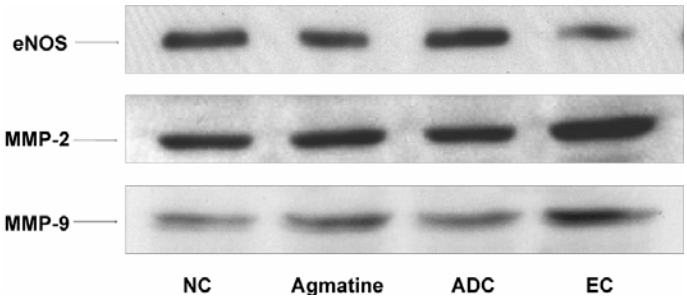


Fig. 11. Agmatine administered exogenously and endogenously modulate the protein expression of *e*NOS, MMP-2 and MMP-9. Cells were exposed to normoxia (NC) or OGD-reperfusion injury (Agmatine, agmatine was added at the start of reperfusion; ADC, ADC overexpression cell; EC, experiment control). Total protein samples were collected and analyzed for MMP-2 and MMP-9 by western blotting.

Activating transcription factor 3 (ATF_3) is rapidly induced in response to a variety of stress such as ischemia reperfusion injury in endothelial cells, suggesting that it plays a role in the stress-responsive pathway[25]. It was shown that the ATF_3 was markedly increased in the ADC overexpressioned cells. We performed a further inhibitory study to evaluate whether the effect is related to the *e*NOS. The result indicated that agmatine endogenously administrated was significant attenuated by a NOS inhibitor. It seems that the ADC overexpression cells increased the ATF_3 expression that is mediated *e*NOS.

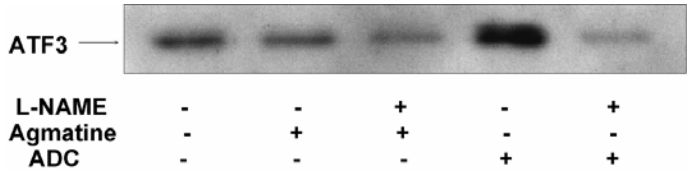


Fig. 12. Effect of agmatine administrated exogenously and endogenously on ATF_3 expression after OGD-reperfusion injury. Results show the effect of combined administration of agmatine and L-NAME on ATF_3 protein expression in bEnd.3 cells and bEnd.3ΔADC cells after OGD reperfusion injury. Agmatine and L-NAME were added to the culture medium at the start of reperfusion.

10. Immunolocalization of ATF3 in bEnd.3 cells

For immunofluorescence, cells were washed with PBS, fixed in 4% paraformaldehyde, permeabilized with 0.05% Triton. After blocking, samples were incubated with ATF_3 antibody. After washing with PBS, goat anti-rabbit IgG (H+L) FITC conjugate was added. Then nuclei were stained with 4, 6-diamidino-2-phenylindole (DAPI). The samples were viewed with a fluorescent photomicroscope. It was viewed that a great part of ATF_3 were stained at the cell nucleus, especially in ADC overexpressioned cells (Fig. 13) (A.EC; B.agmatine; C. ADC).

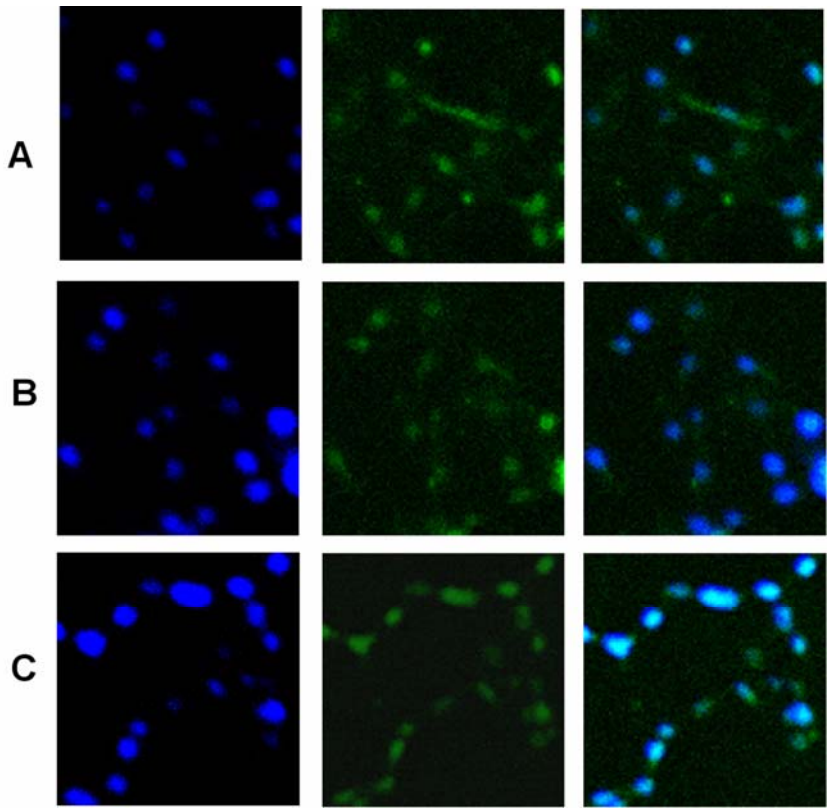
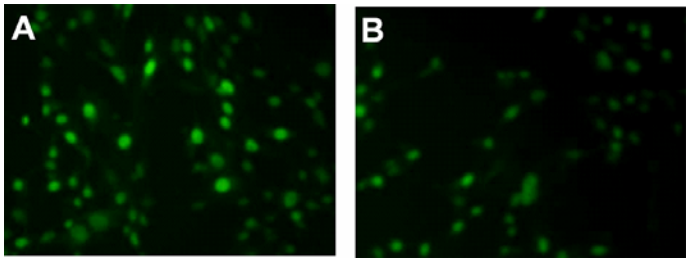


Fig. 13. Agmatine administrated exogenously and endogenously modulate immunolocalization of ATF_3. Cells were immunostained after nomoxia (A), or OGD-reperfusion injury (B. bEnd.3 cells; C. bEnd.3ΔADC cells). Nucleus of the cells were stained with 4', 6'-diamidino-2-phenylindole (DAPI).



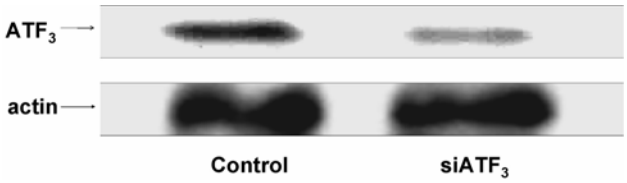
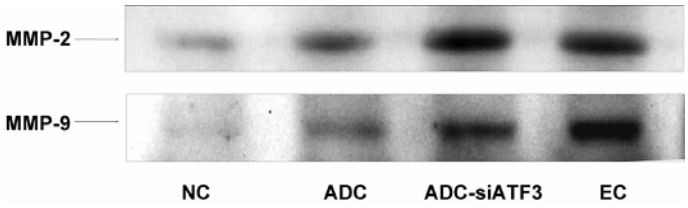


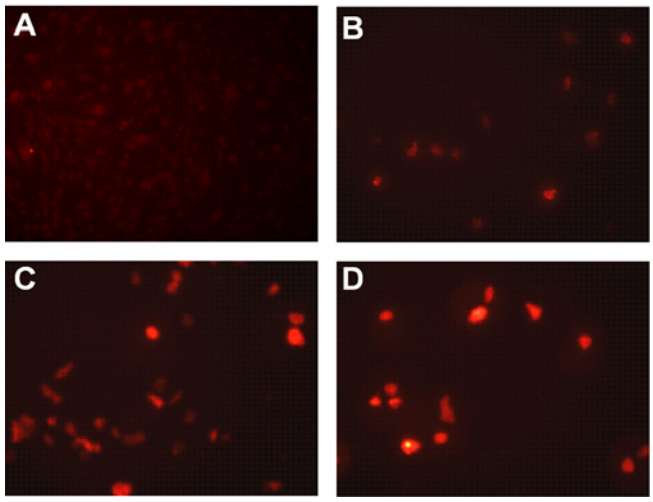
Fig. 14. Blockage of ATF_3 in bEnd.3ΔADC cells. Results of western blot and immunocytochemical analysis showing specific inhibition of ATF_3 expression by siRNA (A. NC; B. $siATF_3$).

11. Blockage of ATF_3 expression by siRNA leads to the increase of MMP-2 and MMP-9 expression in bEnd.3ΔADC cells

To further examine the role of ATF_3 on MMP expression, bEnd.3ΔADC cells were transfected with small interfering RNA (siRNA) designed to suppress ATF_3 expression. Expression of ATF_3 was abolished by transfection of siRNA against ATF_3. Two days after transfection, cells reached confluence, and were exposed to OGD reperfusion injury. Then western blotting and Immunocytochemical analysis were conducted. The expression of ATF_3 was suppressed compared with control (Fig. 14). The expression of MMP-2 and MMP-9 was decreased in ADC overexpressed bEnd.3 cells. But, the MMP-2 and MMP-9 expression were attenuated in cells transfected with ATF_3 siRNA (Fig. 15).



(a)



(b)

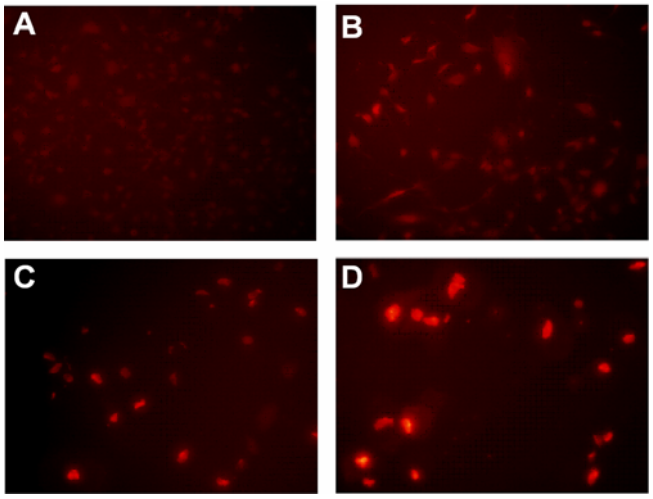


Fig. 15. Blockage of ATF_3 expression by siRNA leads to the increase of MMP-2 and MMP-9 expression in bEnd.3ΔADC cells. Cells were exposed to normoxia (NC) or OGD-reperfusion injury (ADC, ADC overexpression cells; ADC-siATF3, transfected with ATF_3 siRNA cells; EC, experiment control). Cells were analyzed for MMP-2 and MMP-9 by western blotting and immunocytochemical analysis (a. MMP-2; b. MMP-9).

12. Immunohistochemical analysis of MMP-2 and MMP-9 after ischemic injury in the MCAO *in vivo* system

We investigated the effect of agmatine in brain ischemic injured mice by MCAO. Agmatine were injected intraperitoneally into the brain ischemic injured mice. The effect of agmatine on MMP-2 and MMP-9 expression was measured by immunohistochemical analysis. The expression of MMP-2 and MMP-9 was decreased in agmatine administrated compared to experimental control (Fig. 16).

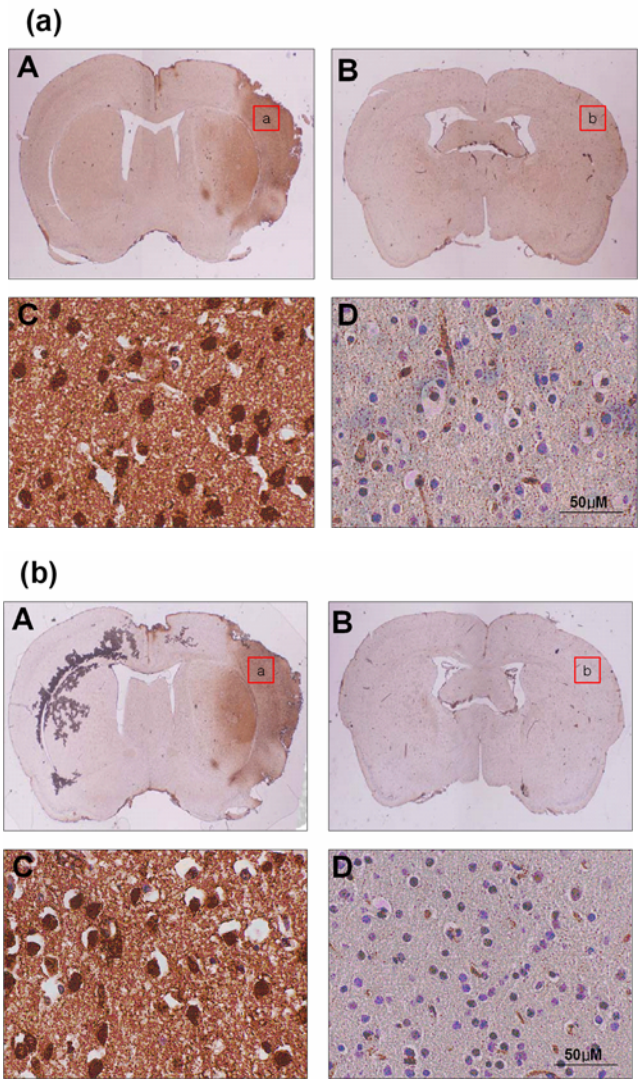


Fig. 16. Immunohistochemical analysis of MMP-2 and MMP-9 in *in vivo* MCAO model. Male ICR mice were subjected to transient middle cerebral artery occlusion (MCAO). The brain were cryosectioned 10-㎛-thick. The immunofluorescence staining for MMP-2 and MMP-9 were carried. The expression of MMP-2 and MMP-9 was decreased in agmatine administrated (B, D) compared to experimental control (A, C).

Ⅳ. Discussion

Ischemia, caused by transient or permanent reduction of cerebral blood flow, is a leading cause of neuronal cell death. Following ischemia, there is a loss of microvascular integrity which is manifested by major alterations in vascular permeability. As a result, the vascular extraccellular matrix is lost, which is a constituent of the basal lamina, which plays a critical role in maintaining the integrity of the BBB on the endothelial cell wall by providing structural supports. Matrix metalloproteinases (MMPs) degrade the basement membrane of brain vesssels to promote cell death and tissue injury. In the present study, we investigated the production of MMP-2 and MMP-9 by CNS microvascular endothelium, and demonstrated that both MMP-2 and MMP-9 are up-regulated by these cells *in vitro* following ischemia insult. We found that the treatment of endothelial cells with agmatine exogenously and endogenously led to MMP-2 and MMP-9 reduction in primary cultured cerebral endothelial cells and bEnd.3 cells. To investigate the modulation of matrix metalloproteinase expression by agmatine in protein level, we performed immunocytochemistry and western blotting. The result showed that immuno-positive cells were reduced in agmatine exogenously administrated group especially in the post-agmatine treatment group compared to the EC group

($p<0.05$) (Fig. 1). We also observed in the western blot analysis that agmatine attenuated the expression of the MMP-2 and MMP-9 induced by ischemic injury in both exogenously and endotgenously treated, especially in endogenously administrated.

Recent studies have shown that agmatine has a neuroprotective effect in *in vivo* and *in vitro* against ischemic injury[31]. However, its physiological action on the brain microvasculature remains unclear. NO and agmatine seem to have some important interactions. Agmatine affects NO synthesis by activating endothelial NOS (*e*NOS)[41, 42] and by inhibiting inducible (*i*NOS)[43, 44] and neuronal NOS (*n*NOS)[45]. NO production by *i*NOS occurs primarily by inflammatory cells and results in high NO levels and toxic products. In contrast, NO production by *e*NOS is known to play an important role in the regulation of vascular reactivity[46], and a protective role within the vascular system by its vasodilatory effect[47]. Radomski et al. suggested that the overproduction of NO in the brain contributes to tissue damage, but a low level NO production by *e*NOS is believed to positively affect the outcome of ischemia by improving ischemic blood flow[48]. Results of the present study show that shortened ischemia-reperfusion injury decreased the concentration of NO production in endothelial cells. The agmatine-treated groups

demonstrated an attenuated decrease of NO compared to the EC group, especially in the post-agmatine treatment group. And it was also observed in agmatine endogenously administrated group. In this experiment, we also observed that the expression of *e*NOS was increased by agmatine administrated exogenously and endogenously. Taken together, agmatine stimulated and increased NO production in endothelial cells, and appeared to act directly on endothelial cells to increase the synthesis of nitric oxide.

The protein and gene regulation of MMPs are complex and NO seem to play a vital role. Eberhardt et al. found that exogenously and endogenously produced NO significantly accelerates the degradation of MMP-9 mRNA[49]. And now we have investigated that the expression of MMPs was suppressed and *e*NOS was increased by agmatine administrated exogenously and endotgenously. So, in a further experiment, we examined whether the MMPs expression was related to the *e*NOS. The result showed that the co-administration of L-NAME (NOS inhibitor) and agmatine did not alter NO production. It suggested that the effect of agmatine on NO reduction was related to the NOS. In addition, the expression of MMP-9 was not down-regulated in the presence of a NOS inhibitor while the expression of MMP-2 did not show same result in the presence of L-NAME. And, it was also shown

in mRNA levels. The present study demonstrated that L-NAME altered the effect of agmatine on MMP-9 expression, but not on MMP-2. It seems that agmatine's inhibition of MMP-9 expression is mediated, at least in part, via *e*NOS and the maintenance of functional NO release in cerebral endothelial cells.

The MMPs are regulated at different levels, including transcriptional level. So, some transcription factors may regulate the MMPs expression. ATF_3 is one of the transcription factor which is rapidly induced in response to a variety of stress such as ischemia reperfusion injury in endothelial cells[22, 23], suggesting that it plays a role in the stress-responsive pathway. Kawauchi et al[24] clearly demonstrated that ATF_3 acts as a transcriptional repressor to protects endothelial cells from tumor necrosis factor-induced apoptosis. It was shown that NO induced a dose and time dependent induction of ATF_3 expression and the ATF_3 induction resulted in the inhibition of MMP-2 promoter activity[25]. Nobori et al demonstrated that ATF_3 has cardioprotective effects and its homodimer was known to repress transcription of several genes[50].

In our experiments we found that ATF_3 was dramatically increased in the bEnd.3ΔADC cells which were administrated agmatine endogenously. Furthermore, it was observed that a great part of ATF_3 were stained at the cell nucleus, especially in ADC overexpression cells compared to control and agmatine administrated exogenously group (Fig. 13). And in a further inhibitory study, we found that the expression of ATF_3 by endogenously administrated agmatine was markedly attenuated by NOS inhibitor. It also seems that the endogenously agmatine increased the ATF_3 expression is mediated *e*NOS. For examine the role of ATF_3 on MMPs expression, bEnd.3ΔADC cells were transfected with small interfering RNA (siRNA) designed to suppress ATF_3 expression. The expression of ATF_3 was suppressed compared with control (Fig. 14). And the suppression of MMP-2 and MMP-9 by endogenously administrated agmatine were attenuated in cells transfected with ATF_3 siRNA (Fig. 15).

In conclusion, present study demonstrated that exogenously and endogenously administered agmatine suppressed the MMP-2 and MMP-9 expression after OGD- reperfusion injury in cerebral endothelial cells. The effect is related to the *e*NOS and the maintenance of functional NO release. And, *e*NOS

and functional released NO induced up-regulation of ATF_3 expression resulted in the inhibition of MMP-2 and MMP-9 expression in bEnd.3ΔADC cells.

Ⅴ. Conclusion

Present study showed the regulation of MMPs by agmatine administrated exogenously and endogenously in mouse cerebral endothelial cells and ADC overexpressioned endothelial cells. These results have led me demonstrate the following conclusions.

1. The expression of MMP-2 and MMP-9 were suppressed by exogenously and endogenously administered agmatine after OGD-reperfusion injury in cerebral endothelial cells.
2. The expression of *e*NOS and NO production were increased by exogenously and endogenously administered agmatine following ischemia-reperfusion injury.
3. Endogenously administered agmatine decreased the cell death after OGD- reperfusion injury.
4. The inhibition of MMP-9 by exogenously administered agmatine is mediated at least in part via the regulation of *e*NOS and functional NO release.
5. The expression of ATF_3 was dramatically increased in ADC overexpressioned bEnd.3 cells.

6. A great part of ATF_3 were stained at the cell nucleus, especially in ADC overexpressioned cells compared to control and agmatine administrated exogenously group.
7. The *e*NOS and functional released NO induced ATF_3 up-regulation that resulted in the inhibition of MMP-2 and MMP-9 expression in ADC overexpressioned bEnd.3 cells.

Taken together, these data suggested that exogenously and endogenously administered agmatine suppressed the MMPs expression after OGD-reperfusion injury in cerebral endothelial cells. The effect is related to the *e*NOS and the maintenance of functional NO release. And, *e*NOS and functional released NO induced up-regulation of ATF_3 that resulted in the inhibition of MMP-2 and MMP-9 expression in ADC overexpressioned bEnd.3 cells.

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ABSTRACT (IN KOREAN)

마우스 내피세포의 허혈 손상에 대한 아그마틴의 효과

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뇌 허혈 손상 시 뇌 미세혈관의 결합이 손상되고, 이에 따라서 혈관내피세포에 의해 지지되고 있는 뇌혈관장벽에 손상을 주게 된다. Extracellular matrix 는 뇌 혈관장벽을 보존하는데 중요한 작용을 하는 물질이며, 뇌 허혈 손상 시 증가된 matrix metalloproteinase 에 의해 분해된다. 혈관내피세포에서 발현되는 *e*NOS 와 이에 의해 생성된 NO 는 혈관기능을 조절하는데 중요한 보호역할을 한다고 보고되었다. 또한 ATF_3 는 전사조절 인자로서 뇌허혈과 같은 스트레스를 받았을 경우 혈관내피세포에서 발현이 증가되며 일부 유전자의 전사를 억제함으로써 보호효과를 보여주고 있다고 한다. 아그마틴은 아르기닌의 탈탄산효소에 의해 L-아르기닌의 탈탄산화에 의해 형성된 1 차 아민으로 이전 연구에서 아그마틴의 신경보호 효과를 이미 보고한 바 있고, 또한 NO 생성과도 연관되어 혈관 기능에 보호효과가 있다고 보고된바 있다. 본 연구에서는 이러한 아그마틴이 뇌허혈 손상 시 MMPs 에 대한

조절작용을 관찰하였으며 그 기전에 대해 알아보았다. 연구 결과, 아그마틴이 MMP-2 와 MMP-9 의 발현은 감소시켰고, *e*NOS 의 발현과 NO 생성은 증가시켰다. 아그마틴에 의한 MMP-2 의 감소는 NOS 길항제인 L-NAME 를 처리 시 아그마틴에 의해 여전히 감소하였으나, MMP-9 의 발현은 감소되지 않았다. 따라서, 아그마틴 처리시 뇌혈관 내피세포에서 MMP-9 의 감소는 *e*NOS 와 이에 의해 생성된 NO 의 증가와 어느 정도 연관성이 있음을 알 수 있었다. 또한 본 연구에서는 아그마틴을 생성하는 효소인 아르기닌의 탈탄산효소를 혈관내피세포에 infection 시켜 내재적인 아그마틴을 생성하게 하였다. 내재적인 아그마틴 생성은 ATF_3 의 발현을 증가시키는 것을 관찰할 수 있었는데, L-NAME 로 처리시 증가를 억제하였다. 또 ATF_3 의 siRNA 를 이용하여 ATF_3 를 억제시켰을 경우 내재적 아그마틴에 의한 MMPs 의 감소효과가 억제됨을 볼 수 있었다. 결론적으로, 아르기닌의 탈탄산효소를 혈관내피세포에 infection 시켜 내재적인 아그마틴을 생성하게 하였을 경우, *e*NOS 의 발현과 NO 생성이 증가되며 이에 의한 ATF_3 발현양의 증가에 의해 MMP-2 와 MMP-9 의 감소를 초래하게됨을 알 수 있다. 따라서, 결론적으로 아그마틴은 뇌허혈 손상 시 혈관내피세포에서 MMP-2 와 MMP-9 의 발현을 억제함으로써 뇌혈관벽의 손상을 막는데 중요한 역할을 한다는 것을 알 수 있었다.

핵심되는 말: 아그마틴, 아르기닌의 탈탄산효소, matrix metalloproteinase, 내피세포의 일산화질소 생성효소, 일산화질소, activating transcription factor 3