Regulation of Nuclear factor-κB nuclear translocation by agmatine in primary cultured astrocytes following ischemic insults

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Regulation of Nuclear factor-κB nuclear translocation by agmatine in primary cultured astrocytes following ischemic insults

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ABSTRACT

Regulation of Nuclear factor-κB nuclear translocation by agmatine in primary cultured astrocytes following ischemic insults

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Agmatine is a primary amine formed by the decarboxylation of L-arginine and is an endogenous clonidine-displacing substance synthesized in mammalian brain. Many studies suggest that agmatine reduces various brain injury. We investigated the protective effect of agmatine in the primary cultured astrocytes under ischemic-like injury. Agmatine protected the astrocytes from necrotic and apoptotic cell death under oxygen-glucose deprivation. Especially, agmatine decreased necrotic cells in OGD phase, but

at the end of OGD-R, agmatine reduced delayed apoptotic cells. These protections were associated with an induction of nuclear factor-κB (NF-κB) nuclear translocation. Western blotting analysis showed that agmatine increased nuclear translocation of NF-κB, which were inhibited by the inhibitor (MG132). Futhermore, agmatine increased the expression of p-IkBα and p-IKKα/β in astrocytes under ischemic conditions. Agmatine also regulated phosphorylation of p42/p44 MAPK (ERK1/2) and c-Jun N-terminal kinase (JNK), which were attenuated by U0126 or SP600125, respectively. Especially, the expression of p-ERK was slightly increased with agmatine, while that of p-JNK was significantly reduced in astrocytes under ischemic injury. However, phosphorylated p38 was not influenced by agmatine. But these MAPKs regulated by agmatine regulated cell death in OGD treated astrocytes. NF-κB nuclear translocation by agmatine was linked with the expression of other anti-apoptotic protein such as Bcl-2, and the Bcl-2 expression was downregulated by MG132 in astrocytes under OGD. These results demonstrated that neuroprotective effect of agmatine seemed to be related with the regulation of Bcl-2 via NF-κB pathway and the regulation of MAPKs expression by agmatine did not seemed to be related with NF-κB translocation directly.

Key words : Agmatine, Oxygen-glucose deprivation, Astrocyte, NF-κB, MAPKs, Bcl-2

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I. INTRODUCTION

Astrocytes play a crucial role in the central nervous system (CNS). They modulate synaptic transmission and plasticity, guide axonal growth during development and secrete growth factors to supprot neuronal survival[1,2,3]. Therefore, dysfunction or loss of astrocytes can lead to neuronal death or dysfunction[4,5,6]. Since astrocytes play a central role in maintaining neuronal viability both under normal conditions and during stress such as ischemia, its function to stress is essential to understand various brain pathology. A significant amount of brain damage caused from ischemic stroke can be attributed to neuronal cell death, resulting from an insufficient

supply of glucose and oxygen to brain tissue[7]. To understand the mechanisms of neuronal cell death after ischemic insult and to identify potential protective agents, in vitro cell culture model of ischemia has been developed[8]. The experimental paradigm includes an initial short phase of oxygen and glucose deprivation (OGD) followed by a prolonged phase of restoration (adding back oxygen and glucose, OGD-R). The OGD phase mimics the lack of oxygen and glucose supply, such as a thrombus formation during stroke, while the OGD-R phase reflects the restoration of oxygen and glucose supply to the injured brain.

Agmatine, a naturally occurring guanidino compound found in abundance of bacteria and plants was recently identified in mammalian brains[9,10]. Agmatine is formed from decarboxylation of L-arginine by the arginine decarboxylase (ADC)[11]. It is hydorolyzed to putrescine and urea by agmatinase[12,13]. Agmatine is an endogenous clonidine-displacing substance[14], an agonist for the α2-adrenergic and imidazoline receptors[15], and an antagonist for the N-methyl-$_D$-aspartate (NMDA) receptors[16]. Recent studies have shown that agmatine may be neuroprotective in neurotrauma, neonatal ischemia models and cultured neurons[17,18]. Agmatine is synthesized, stored in astrocytes, and released from specific networks of neurons[19]. However, the molecular mechanisms of agmatine action in astrocytes are still unknown.

The typical transcription factor NF-κB plays an important physiological and pathological role in a variety of tissues and cells, including brain cells[20]. In astrocytes, NF-κB activity is required for the inducible expression of various genes involved in the pathogenesis of cerebral ischemia. NF-κB complexes are mainly composed of p65 and p50 subunits in astrocytes[21,22], and remain sequestered in the cytoplasm of resting cells by association with a family of inhibitory IκB proteins. Following the appropriate stimuli, the IκB proteins are rapidly

phosphorylated[23] by the IκB kinase complex (IKK), ubiquitinated, and degraded by the 26 S proteasome[24]. As a result, NF-κB translocates to the nucleus to bind specific κB DNA motifs and promote expression of target genes[24]. Recent attempts to identify the upstream kinase of IKK have revealed the involvement of MEK kinase 1 (MEKK1)[25,26], and NF-κB-inducing kinase (NIK)[27]. In addition, NF-κB activity is highly related to mitogen-activated protein kinases (MAPKs) pathways in many signal cascades. Nevertheless, the molecular mechanisms of how MAPKs pathway contribute to the activation of NF-κB in astrocytes remain to be characterized. This suggests that protective effect of agmatine can be related to MAPKs and NF-κB pathway in OGD-R injury. In this study, we investigated the protective effects of agmatine and the role of MAPKs and NF-κB regulated by agmatine in OGD-R stimulated astrocytes.

II. MATERIALS AND METHODS

1. Materials

Polyclonal anti-NF-κB-p65 antibody and monoclonal anti-phospho p38 and actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-phospho-IKKα/β, phospho-IκBα, phospho-JNK and phospho-p42/p44 MAPK were obtained from Cell Signal Technology (Beverly, MA, USA). Horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence (ECL) western blotting detection system were obtained from Amersham Biosciences (Uppsala, Sweden). Other inhibitors were obtained from Sigma (St. Louis, MO, USA).

2. Astrocyte cultures

Primary cortical astrocytes were cultured from 1-to 3-day old postnatal ICR mouses and maintained in Minimum essential medium (MEM, Gibco) containing 10% fetal bovine serum and 10% equine serum (Hyclone). Briefly, hemispheres of newborn ICR mouses were removed aseptically from the skulls, freed of the menings. And cortex was dissected, treated with 0.09% trypsin for 20 min at 37℃, and triturated. Cells were plated onto 6-well and 24-well plates at a density of 1 hemisphere per plate and the culture medium was changed twice a week for 20 days.

3. Oxygen-glucose deprivation

To initiate oxygen-glucose deprivation (OGD), cultures were transfered into an anaerobic chamber (Forma Scientific, OH, USA) (O_2 tension < 0.1%), washed three times with deoxygenated, glucose-free balanced salt solution (BSS_0) containing 116mM NaCl, 1.8mM $CaCl_2$, 0.8mM $MgSO_4$, 5.4mM KCl, 1mM NaH_2PO_4, 14.7mM $NaHCO_3$, 10mM

HEPES and 10mg/L phenol red at pH 7.4 and incubated in BSS_0 at an oxygen-free incubator for 4 h. Following OGD, glucose added to culture medium to a final concentration of 5.5mM and cells were incubated under normal growth conditions for indicated time.

4. Measurement of Lactate dehydrogenase (LDH) activity

Cell lysis was quantified by assay of LDH activity released into the culture medium[28]. Total LDH release, corresponding to complete cell death, was determined at the end of each experiment after freezing at -70 ℃ and rapid thawing.

5. Hoechst-PI nuclear staining

The cell death was evaluated by staining of non-viable cells with propidium iodide (Sigma), and live cells with Hoechst 33258 dye (Sigma). Staining with the fluorescent dyes propidium iodide and Hoechst 33258 allows discrimination of apoptotic cells on the basis of nuclear morphology and evaluation of membrane integrity. Hoechst dye was added to the culture medium to a final concentration of 2-5 μg/mL and the cultured cells were kept at 37℃ for 30min. Propidium iodide solution was then added (final concentration 2-5 μg/mL) just before observation in a Olympus microscope equipped for epifluorescence with UV filter block.

6. Flow cytometry analysis of cell death

Apoptosis was determined using an apoptosis detection kit (BioVision Inc., CA. USA). Briefly, cells were collected after treatment, washed twice in ice-cold PBS, and then resuspended in binding buffer at a density of 1×10^5 cells/mL. Cells were incubated simultaneously with FITC-labled annexin V and propidium iodide (PI) for 10min. Flow cytometry was

performed to determine the populations positive for annexin V-FITC (FL-1 channel) and/or PI (FL-2 channel) by a FACScan (Becton Dickinson, Mountain View, CA). Data were analyzed using Cell Quest software (Beckton Dickinson Immunocytometry Systems).

7. Preparation of cytosolic and nuclear extracts

Primary cultured astrocytes were washed with ice-cold PBS and harvested cells were solubilized with buffer A [10mM HEPES, pH 7.4, 10mM KCl, 0.1mM EDTA, 0.1mM EGTA, 1mM DTT, 1μg/mL leupeptin, 1 μg/mL aprotinin, 1mM PMSF]. After 15min on ice, Igepal were added to the lysates to a final concentration 0.5%. The tubes were vigorously vortexed for 10sec and the nuclei collected by centrifugation at 14000 rpm for 30sec. The supernatants were stored at -80℃(cytosolic fraction), and the nuclei pellet was resuspended with buffer B [20mM HEPES, ph 7.4, 400mM NaCl, 1mM EDTA, 10% glycerol, 1mM DTT, 1μg/mL leupeptin, 1 μg/mL aprotinin, 1mM PMSF]. The nuclei solution incubated on ice for 30min vortexing every 10min, nuclear proteins were obtained by centrifugation at 14000rpm for 15min, and the supernatants stored at -80℃. Protein concentration was quantified using the BCA protein assay kit (Pierce, Rockford, IL, USA).

8. Western blot analysis

Equal amounts of protein were subjected to electrophoresis on 10% SDS-polyacrylamide gels. Separated proteins were then electro-transferred toImmobilion-NC membrane (Millipore, Bedford, MA, USA). The membranes were blocked for 1h at room temperature in 5% skim milk in TBS plus 0.1% Tween-20 (TBS-T). The membranes were incubated overnight with anti-NF-κB-p65, anti-phospho(Ser32) IκBα, anti-IκBα,

anti-phospho IKKα(Ser180)/β(Ser181), anti-phospho p42/p44 MAPK, anti-phospho JNK, and anti-phospho p38. After washing 3 times with TBS-T for every 5min, blots were incubated with peroxidase-conjugated anti-rabbit or anti-mouse secondary antibodies for 1h at RT. Finally, blots were rinsed and proteins were visualized using an ECL protein detection kit according to the manufacturer's instructions.

9. Statistical analysis

Results are presented as the mean ± SEM of at least three different experiments performed in separate cell preparation, duplicate or triplicate determination were performed in each experiments. One-way ANOVA followed by Student's t-test was used as indicated in order to examine the statistical significance; p-values less than 0.05 were considered significant.

III. RESULTS

1. Protective effect of agmatine on OGD-R injury

To investigate the protective effect of agmatine on OGD-R injury, primary cultured astrocytes were subjected to OGD 4h and restoration up to 20h in the absence or presence of agmatine (100μM). Effect of agmatine was analyzed by Hoechst-PI nuclear staining. Fig. 1A shows the results of representative experiment out. Hoechst-stained nuclei (blue) of astrocytes after OGD-R injury were gradually decreased following restoration time-dependent manner. In contrast, PI-stained nuclei (red) increased by OGD-R injury. However, agmatine treated astrocytes had more Hoechst-stained nuclei in comparison with cells receiving no treatment. To further examine this phenomenon, we carried out assay of lactate dehydrogenase (LDH) release. Fig. 1B shows the effect of agmatine on LDH release into the culture medium. In normoxic cells, LDH level in the medium was very low (3.59%). In OGD-R subjected cells, LDH level in the medium was gradually increased to 36.85%. However, treatment with agmatine caused an attenuation of LDH release in astrocytes under OGD (approximate 12% of LDH level; $p<0.01$, as compared with the agmatine untreated level in the end of OGD-R). These results suggest that agmatine may be involved in protective effect of astrocytes under OGD-R injury.

2. Role of agmatine on OGD-R induced necrosis and/or apoptosis

To determine function of agmatine on necrosis and/or apoptosis induced by OGD-R, astrocytes were stained with both PI and FITC-AV, then analyzed by flow cytometry. Necrotic cells are demonstrated by AV^-/PI^+ staining, since PI enters cells when membrane integrity is lost and binds nucleic acids. Apoptotic cells are demonstrated by AV^+/PI^- staining,

since annexin V binds to phosphatidylserine that translocates to the outer side of the plasma membrane during apoptosis. AV^+/PI^+ stained cells are likely to be late apoptotic or necrotic and AV^-/PI^- cells represent viable cells. Fig. 2 shows representative dot plots of cells stained with PI and FITC-AV. OGD 4h resulted in a significant increase in the percentage of necrotic cells (AV^-/PI^+) from a control value of 15.6 to 38.2%. Few apoptotic cells (AV^+/PI^-) were detected. At the end of OGD-R, 31.6% of the cells population was late apoptotic and necrotic compared to 19.8% of the normoxic cells. These results indicate that OGD caused necrosis and late apoptosis occured during the OGD-R. To determine whether agmatine reversed either necrotic or late apoptotic cell death, astrocytes were exposed to OGD and OGD-R. Agmatine reduced necrosis at OGD period, while, at the end OGD-R, reduced late apoptosis and necrosis.

3. Effect of agmatine on localization of NF-κB into the nuclei

To determine the regulation of NF-κB-p65 translocation to the nuclei by agmatine, we performed western blot analysis of both the cytosolic and the nuclear fractions harvested from OGD-R stimulated astrocytes treatment with 100μM agmatine. Fig. 3A shows effect of agmatine on NF-κB-p65 nuclear translocation induced by OGD-R injury. In normoxic cells, NF-κB-p65 proteins were mainly localized in the cytosol. In cells under OGD 4h and restoration up to 2h, however, nuclear translocation of NF-κB-p65 was apparent, reaching maximal levels before restoration phase was started. Furthermore, administration of agmatine increased the amount of NF-κB-p65 detected in the nuclear extracts. Consistent with these results, IκBα levels of cytosol were decreased in agmatine treated cells, while p-IκBα levels were increased (Fig. 3B). Since IκBα proteins regulated by IκB kinase (IKK) complex, we measured the expression of p-IKKα/β by western blot analysis.

As shown in Fig. 3B, in OGD treated astrocytes, expression of p-IKKα/β was increased equal to NF-κB-p65 nuclear translocation. In agmatine treated astrocytes p-IKKα/β and p-IκBα were increased. These results suggest that agmatine may be involved in NF-κB-p65 nuclear translocation induced by OGD-R through regulation of p-IKKα/β.

4. Role of agmatine on phosphorylation of MAPKs

Since MAPKs were known to be related to neuronal protection, we investigated the roles of agmatine to the phosphorylation of MAPKs in OGD-R induced astrocytes. Fig. 4 shows the expression level of phosphorylated MAPK family members by western blot analysis. OGD-R injury induced expression of p-ERK, p-JNK and p-p38 at the different time point. In normoxic cells, p-ERK, p-JNK and p-p38 were not mainly expressed. In OGD-R induced cells, however, the expression of p-ERK gradually were increased at restoration time-dependent manner and were expressed maximum levels at the end of OGD-R. Agmatine slightly increased p-ERK under OGD-R. In case of JNK, the expression of p-JNK increased by OGD 4h, and rapid decreased. However, agmatine markedly decreased p-JNK levels under OGD 4h. p-p38 also increased by OGD 4h, and prolonged to restoration 2h. The expression of p-p38 had no changes with agmatine treatment. These data suggest that NF-κB-p65 nuclear translocation by agmatine may be involved with expression of p-ERK and p-JNK.

5. Roles of ERK and JNK regulated by agmatine on the phosphorylation of IκBα

We investigated the roles of ERK and JNK regulated by agmatine on NF-κB-p65 nuclear translocation in astrocytes under OGD-R. Fig. 5

presents the effect of agmatine on p-ERK and p-JNK contributed to the expression of p-IκBα by western blot analysis. In normoxic cells, we observed very low density of p-ERK, p-JNK, and p-IκBα. To investigate the regulation of MAPK activity through NF-κB pathway, MEK inhibitor U0126 (10μM), specific JNK inhibitor SP600125 (10μM), and 26S proteasome inhibitor MG132 (50μM) were treated in primary cultured astrocytes under OGD 4h. Simultaneously co-administration of agmatine with U0126 caused an slightly increase of p-ERK. However, almost no changes were observed in the phosphorylation of p-IκBα when the cells were exposed to U0126. We also observed that the expression of p-ERK did not influenced by treatment of MG132. In case of JNK, lower levels of p-JNK were detected with co-administration of agmatine and SP600125, similar with SP600125 alone, and treatment of SP600125 did not influenced the expression of p-Iκ Bα. However, p-JNK expression was increased by treatment of MG132, while it was diminished when the cells were exposed to MG132 and agmatine, together. Also co-administration of agmatine and MG132 induced an significantly increase of reduced phosphorylation status of p-IκBα by MG132 alone. These results suggest that the regulation of expression of p-I κBα by agmatine seems to be indirectly linked with JNK-dependent pathway in astrocytes during ischemic injury and the ERK pathway have not been contributed to the expression of p-IκBα.

6. Roles of ERK, JNK and NF-κB regulated by agmatine on the cell death of astrocytes

To investigate the roles of ERK, JNK and NF-κB-p65 regulated by agmatine on cell death, we measured the released LDH to the medium following OGD-R injuries (Fig. 6). In control cells, we observed very low percents of LDH release (7.3%). OGD 4h and R 20h increased the LDH

release to 32.4%. The slight increase in LDH release was observed after an additional exposure of astrocytes to 10μM U0126 under OGD-R (39.8%). This observation appeared an apposite to exposure with 10μM SP600125. SP600125 reduced the increased LDH levels by OGD-R to 18.2%. Ischemic cells treated with 50μM MG132 increased the released LDH levels (49.7%). However, agmatine reduced the percents of LDH release in the presence of U0126 or MG132 to 21.8% or 24.5%, respectively. There were similar results on agmatine treatment in the absence or presence of SP600125. These data suggest that ERK and NF-κB-p65 act in cell survival while JNK acts in cell death, and agmatine may protect cell death through regulation of ERK, NF-κB-p65 and JNK at least in part.

7. Effect of agmatine on the expression of anti-apoptotic proteins, Bcl-2

Following above results, agmatine induced NF-κB-p65 nuclear translocation through the activation of IKKα/β and IκBα. We investigated post-transcriptional regulation of anti-apoptotic proteins by NF-κB-p65 activation. For this purpose, the western blot analysis of anti-apoptotic protein Bcl-2 was performed. Fig. 7A shows that in agmatine-non stimulated cells, Bcl-2 expression is increased by OGD 4h and R 6h, and agmatine helped more increase of Bcl-2 protein levels. Since Bcl-2 are regulated by NF-κB or JNK in apoptosis and agmatine regulates NF-κB-p65 and JNK, Bcl-2 expression may be regulated by NF-κB or JNK in astrocytes under OGD-R injuries. We investigated the role of NF-κB-p65 and JNK on Bcl-2 expression in the presence of agmatine. As shown in Fig. 7B, exposure of MG132 in OGD stimulated astrocytes reduced expression of Bcl-2. However agmatine treatment increased Bcl-2 protein levels in the presence of MG132. SP600125 treatment did not influenced on Bcl-2 expression. These results demonstrated that the regulation of Bcl-2 by agmatine was related with NF-

κB-p65 pathway.

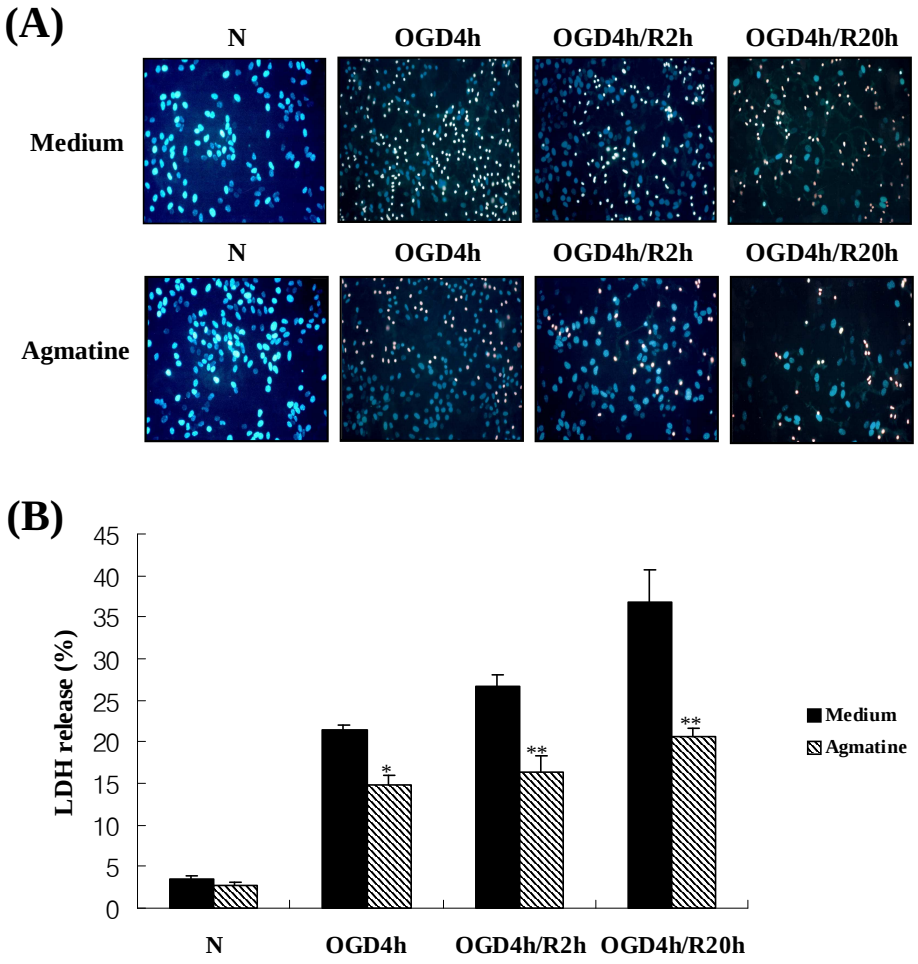

Figure 1. Protective effect of agmatine on the primary cultured astrocytes after ischemic injury. (A) Microphotographs of primary cultured astrocytes stained with Hoechst (Blue)-propidium iodide (Red) after OGD-R in the absence (medium) or presence of agmatine (100μM) administration and 20× objective. Agmatine treatment reduced PI-positive cells induced OGD-R. (B) Effect of agmatine on LDH release in primary cultured astrocytes exposed to normoxia or OGD-R. The results are presented as a percentage value. Each value is indicated as mean ± SE of 4 cultures; *p<0.05, **p<0.01 vs. agmatine un-stimulated group in OGD-R. N, normoxia.

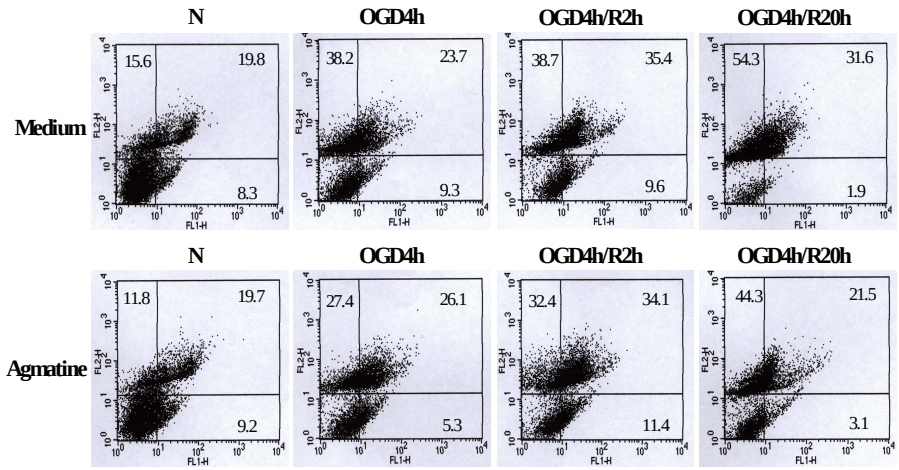

Figure 2. Representative dot plots of flow cytometry of cell death in astrocytes in the absence or presence of agmatine. Astrocytes left untreated (Normoxia) were subjected to 4h of OGD followed by 2h and 20h of restoration (OGD-R) in the absence or presence of agmatine (100μM). Cells were stained with propidium iodide (PI) and FITC-Annexin V (AV). Samples were analyzed by flow cytometry. The numbers above are percentage value. N; normoxia.

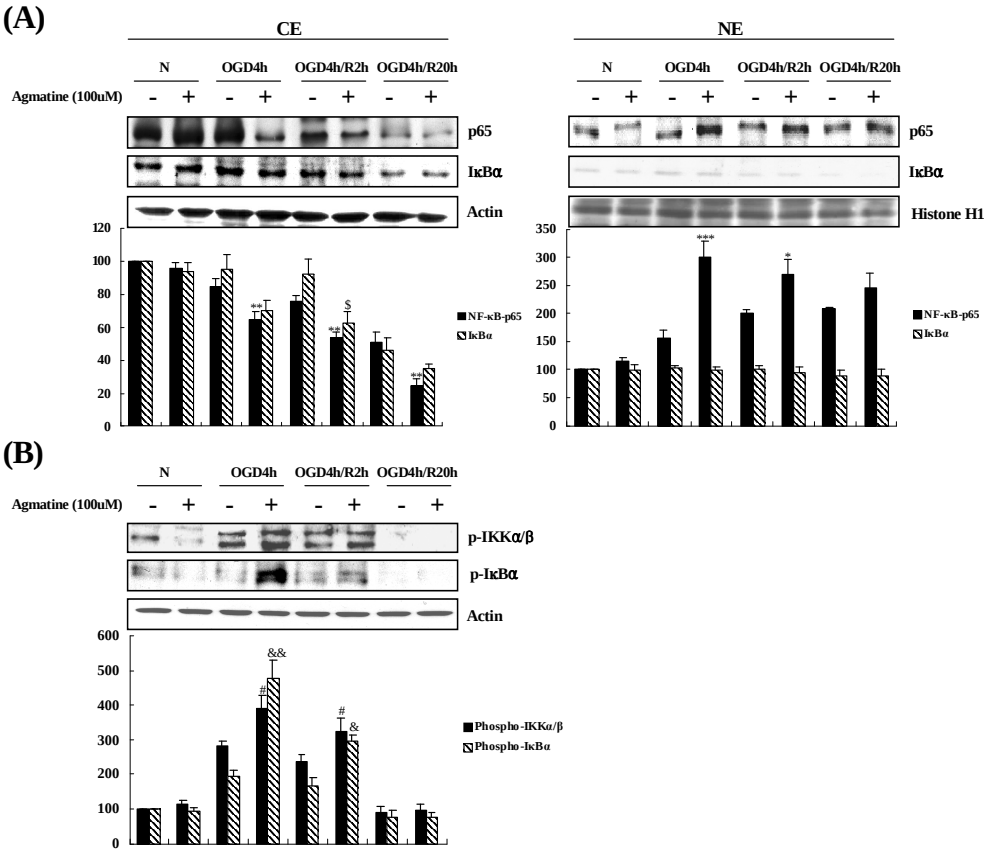

Figure 3. Effect of agmatine on the NF-κB nuclear translocation under OGD-R. (A) Western blot analysis of nuclear NF-κB after OGD-R and treatment in the absence or presence of agmatine (100μM). Fractionated cell lysates (40μg of protein) from experimental groups were subjected to 10% SDS-PAGE and transferred to PVDF membrane, and then blotted using antibodies against p65 and IκBα. Membranes were stripped and reproved with actin or histone H1 as a control. Bands were visualized by an ECL method. (B) Regulation of p-IKKα/β and p-IκBα expression by agmatine under OGD-R injury. *p<0.05, **p<0.01, ***p<0.001 vs. agmatine un-stimulated OGD-R group (p65); $^{\$}p$<0.05 vs. OGD-R alone (IκBα); $^{\#}p$<0.05 vs. OGD-R (p-IKKα/β); $^{\&}p$<0.05, $^{\&\&}p$<0.01 vs. OGD-R (p-IκBα). NE, nuclear extract; CE, cytosolic extract; N, normoxia.

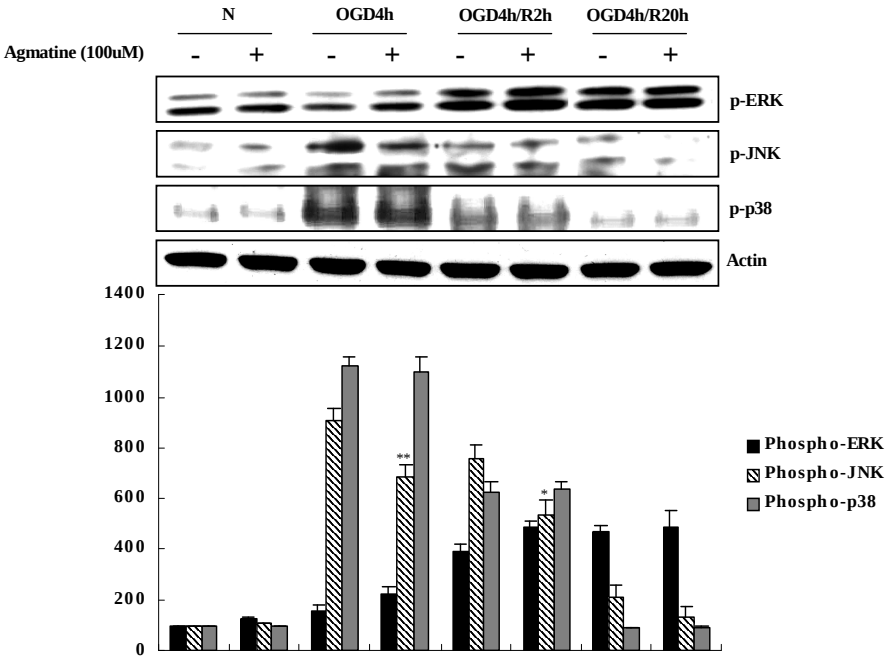

Figure 4. Effect of agmatine on the phosphorylation of MAPKs under OGD-R. Western blot analysis of p-ERK, p-JNK and p-p38 after OGD-R and treatement in the absence or presence of agmatine (100μM). Cell lysates (40μg of protein) from experimental groups were analyzed by western blotting using antiboies against p-ERK, p-JNK and p-p38. *p<0.05, **p<0.01 vs. agmatine un-stimulated group in OGD-R. N, normoxia.

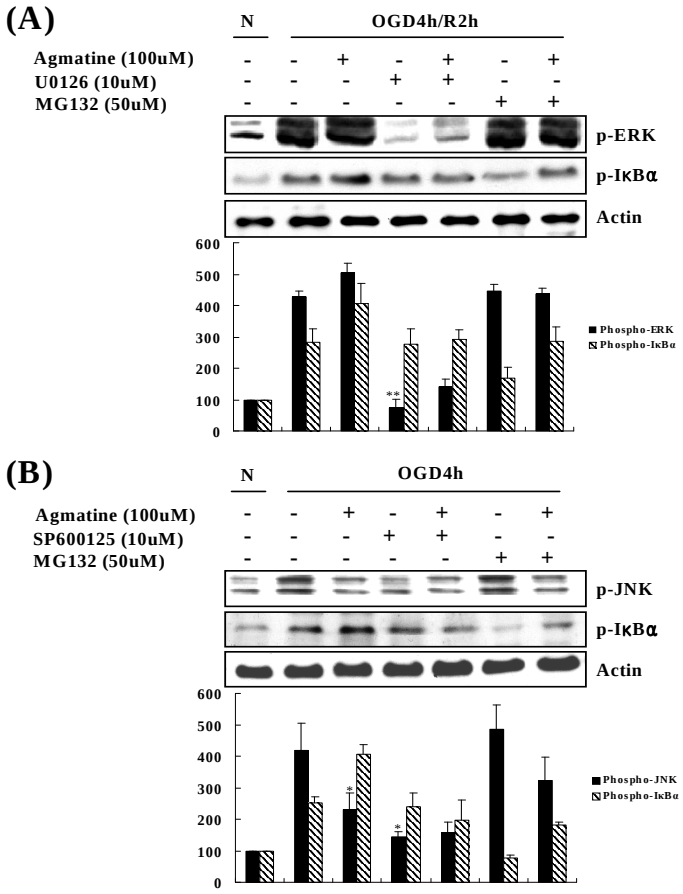

Figure 5. Roles of ERK and JNK regulated by agmatine on the phosphorylation of IκBα. (A) Effect of agmatine (100μM) on expression of p-ERK and p-IκBα in the absence or presence of U0126 (10μM) or MG132 (10μM) exposure were analyzed by western blotting as described in Fig. 3. (B) Effect of agmatine on expression of p-JNK and p-IκBα in the absence or presence of SP600125 (50μM) or MG132 (10μM) exposure were analyzed by western blotting. **p*<0.05, ***p*<0.01 vs. agmatine un-stimulated group in OGD-R. N, normoxia.

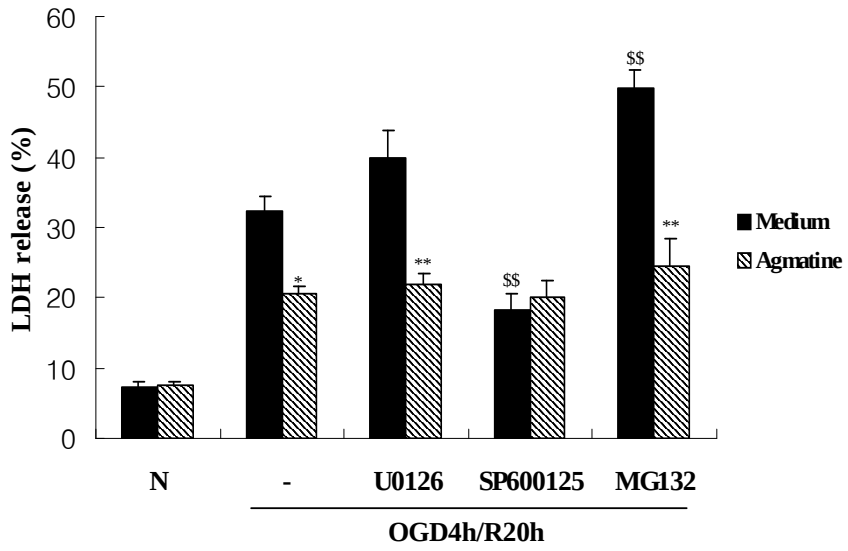

Figure 6. Roles of ERK, JNK and NF-κB regulated by agmatine on LDH release. Astrocytes were treated with U0126 (10μM), SP600125 (10μM) and MG132 (50μM), separately in the absence (medium) or presence of agmatine (100μM). LDH release determined as described above; *p<0.05, **p<0.01 vs. agmatine un-stimulated group in OGD injury; $^{\$\$}p$<0.01 vs. OGD injury alone. N, normoxia; NE, nuclear extract.

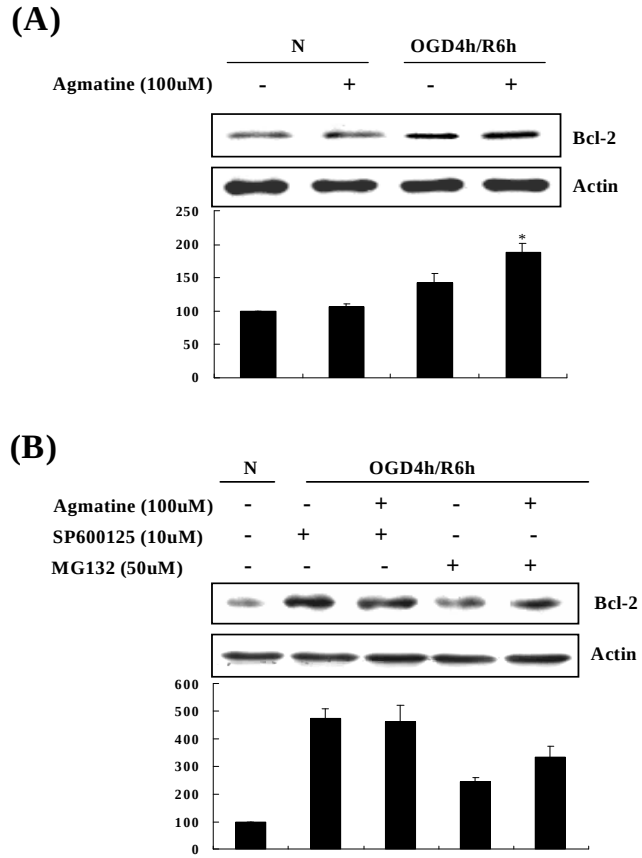

Figure 7. Effect of agmatine on expression of Bcl-2 proteins. (A) Western blot analysis of Bcl-2 expression in OGD-R treated astrocytes. (B) Western blot analysis of Bcl-2 expression in the absence or presence of SP600125 (10μM), MG132 (50μM) and agmatine (100μM). **p*<0.05 vs. agmatine un-stimulated group in OGD-R. N; normoxia.

IV. DISCUSSION

Here we characterize neuroprotective effect of agmatine in astrocytes exposed to OGD-R. Following previously reports, agmatine decreased infarct sizes in middle cerebral artery occlusion mouse model, and promotes survival in neurons exposed to OGD[18]. Agmatine and its synthesizing enzyme, arginine decarboxylase (ADC) are synthesized and stored in astrocytes. Nevertheless, the mechanism of agmatine action is not clear at present in astrocytes itself. We have demonstrated that incubation of astrocytes with agmatine significantly reduces the cell death by OGD-R as measured by the accumulation of LDH in the medium. This reduction in LDH accumulation is consistent with lower PI-stained nuclei in agmatine-treated astrocytes as measured by the Hoechst-PI nuclear staining. Especially, agmatine reduces early necrosis upon exposure to OGD followed by delayed apoptosis during exposure to OGD-R. Both necrosis and apoptosis were precisely quantified using PI and annexin V dual staining.

Knowing that astrocytes cell death induced by OGD-R involved apoptosis, we focused on the role of NF-κB/Rel family members in the death process. NF-κB is an inducible transcription factor composed of various combinations of NF-κB/Rel family members. These proteins include p50 (NF-B1), p52 (NF-B2), p65 (RelA), p68 (c-Rel) and RelB. NF-κB regulate an array of host genes controlling immune activation, inflammation, and the prevention of cellular apoptosis[29,30]. NF-κB is activated by cerebral ischemia in neurons and glia, but its function is controversial. Several studies have found NF-κB can promote neuronal cell death *in vitro*[31,32]. Other investigators have found that inhibition of astrocytic NF-κB had no effect on infarct size in IκBα-superrepressor mice, but neuronal NF-κB activation contributed to cell death[33]. In this study, however, we showed

that treatment of agmatine resulted in a induction of NF-κB nuclear translocation following IKK activation. Also the protective effect of agmatine was demonstrated to be NF-κB-dependent, since LDH accumulation at the end of OGD-R was increased by 26S proteosome inhibitor. These results support the importance of the NF-κB in mediating cell survival, as suggested by a previous in vitro study of GSK-3β-induced apoptosis in astrocytes[34] and OGD-induced cell death in cerebral endothelial cells[35].

The MAPKs also regulate a diverse array of functions, such as neuronal survival and ischemic apoptosis[36,37]. Members of the MAPK family include extracellular signal-regulated kinase (ERK), c-Jun NH2-terminal protein kinase (JNK) and p38 MAPK. JNK and p38 MAPK are strongly activated by stress stimuli, bacterial lipopolysaccharide (LPS) and cytokines, and have been suggested to contribute to cell death[38]. ERK, on the other hand, is activated by mitogenic stimuli, subsequently modulating the activity of many transcriptional factors, leading to proliferation and differentiation[36]. In general, ERK activation has been coupled to protective mechanisms. Neuronal ERK have been implicated in protection against neuronal cell death *in vitro*[39] and *in vivo*[40]. We have found ERK, JNK and p38 of phosphorylation are increased by OGD-R. However, agmatine increased the expression of p-ERK at the delayed phase of OGD-R while decreased p-JNK expression at the early phase of OGD-R. These results demonstrated that regulation of ERK and JNK by agmatine are difference following OGD-R phase. We have found that ERK and JNK might act as a regulator of cell death using MEK inhibitor and specific JNK inhibitor, respectively. We also have found treatment of MEK inhibitor increased LDH accumulation, whereas treatment of JNK inhibitor decreased LDH accumulation. We also have found agmatine can not regulate p38, but function of agmatine on the p38 regulation need to be further investigated.

The suppression of cell death by NF-κB is mainly a transcriptional event. NF-κB-regulated genes that are capable of blocking apoptosis have been identified[41]. Interestingly, the NF-κB-activated pro-survival program appears to be specifically tailored for each tissue and biological context. In some circumstances, the genes are most relevant to the NF-κB anti-apoptotic activity seem to have been identified. For instance, several studies indicate that in peripheral B and T lymphocytes, NF-κB pro-survival signaling induced by antigen receptor and CD40 or CD28 costimulation targets members of the Bcl-2 family such as Bcl-xL, Bfl-1/A1 and Bcl-2 itself[42,43,44]. Indeed, we have found agmatine induces the Bcl-2 protein expression via NF-κB signaling cascade. Since Bcl-2 family members have been linked to mitochondrial function during inhibition of apoptosis, the role of agmatine as a modulator of mitochondrial membrane potential need to be further investigated.

The relation between MAPKs and NF-κB in brain ischemia is intriguing. We attempted to test whether agmatine-mediated NF-κB-p65 nuclear translocation is associated with its ability to regulate ERK or JNK signaling, since there is evidence showing an involvement of MAPKs signaling in activation of NF-κB[25,26,45]. We did not find that ERK pathway contributed to NF-κB-p65 nuclear translocation. However, we have found that JNK pathway might be related with NF-κB-p65 pathway. Especially, it has been suggested that NF-κB-p65 regulated by agmatine down-regulated JNK activation. Since we use 26S proteosome inhibitor which is not high specificity to NF-κB signaling, we have to find whether MAPKs signaling regulated by agmatine is involved NF-κB pathway in astrocyte under OGD-R using by specific NF-κB inhibitor and MAPK kinase inhibitor.

Several groups have shown that there is a crosstalk between the NF-κB and JNK pathway[46,47]. The pro-apoptotic role of JNK is evident from

analyses of JNK-knockout mice. Mouse embryonic fibroblasts lacking both JNK1 and JNK2 are resistant to apoptosis induced by various stress stimuli, and JNK3-/- neurons have severely impaired apoptotic responses to excitotoxins[48]. Inhibition of JNK signaling by pharmacological agents or dominant-negative kinase mutants effectively rescues NF-κB-deficient cells from TNF-α-induced death[46,47]. Likewise, knocking out MKK7/JNKK2 virtually abrogates TNF-α-induced death in RelA-null cells[49]. In this study, we suggest that one of protective mechanisms of agmatine might be through the inhibition of pro-apoptotic JNK pathway.

The relevance of the JNK cascade to apoptosis signaling is highlighted by the finding that activation of this cascade is controlled by NF-κB. Indeed, suppression of NF-κB by ablation of RelA or IKKß leads to persistent JNK induction by TNF-α[46,47]. In short, the containment of the JNK cascade is crucial for the control of TNF-α-induced apoptosis, and this critically depends on NF-κB. The importance of this antagonistic crosstalk between NF-κB and JNK has recently been documented in animal models. Several investigators have shown that NF-κB activation is required to antagonize hepato-toxicity induced by systemic challenge with LPS or concanavalin A (ConA) - two agents that induce liver damage through TNFR-induced cell death[50]. It is plausible that the pro-survival activity of NF-κB in the fetal liver may also involve attenuation of pro-apoptotic JNK signaling.

There are multiple signaling pathways leading to the phosphorylation of IκB and the activation of NF-κB. Modulation of IκB activity are involved in the activation of IKK. Several reports suggest that various upstream activating kinases elevate the IKK activity. These candidates include the MEKK1, MEKK2 and 3, Akt, NIK, atypical PKC (zeta) and 90-KDa ribosomal S6 kinase ($p90^{RSK}$)[51]. We have found agmatine regulates

phosphorylation of IKK, but the regulation of IKK upstream kinase activity by agmatine remains unclear. Since the pivotal role of Akt is a defense the cell death, further studies have to reveal the regulation of Akt expression modulated by agmatine.

In this study, we have demonstrated the protective mechanism of agmatine may be associated with the induction of NF-κB nuclear translocation and regulation of two MAPKs activity in astrocytes after OGD-R. Our results demonstrate the central role played by agmatine in rescuing cell death induced by OGD-R through a induction of NF-κB nuclear translocation. We suggest that the protective effect of agmatine on the astrocytes under OGD-R might be related with the regulation of Bcl-2 through NF-κB translocation into the nucleus. The present study may be helpful for understanding the signaling pathway mediated by agmatine in the astrocytes under brain ischemia.

V. CONCLUSION

This present study showed the role of agmatine on cell death induced by OGD-R. These results have demonstrated following conclusions.

1. Agmatine protect the astrocytes from cell death induced by OGD-R compared to the non-treatment group in LDH assay, Hoechst-PI nuclear staining. Agmatine decreased necrotic cells from a experimental control in OGD phase, but at the end of OGD-R, agmatine reduced delayed apoptotic cells compared to agmatine un-treated group.
2. Agmatine increased NF-κB-p65 nuclear translocation in astrocytes exposed to OGD-R through inducing of IKK phosphorylation.
3. Agmatine also increased the expression of p-ERK, whereas it decreased the expression of pro-apoptotic p-JNK.
4. Agmatine regulated the expression of p-JNK via NF-κB-p65 pathway, but the expression of p-ERK was not related with NF-κB-p65 pathway.
5. Neuroprotective effect of agmatine seems to be related with increase of Bcl-2 protein levels via NF-κB-p65 pathway.

We acertained the protective mechanism of agmatine in astrocytes under OGD-R through NF-κB and ERK/JNK pathways. Also we found the induction of Bcl-2 expression by agmatine is involved with NF-κB pathway.

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ABSTRACT (IN KOREAN)

허혈손상시 별아교세포에서의 아그마틴에 의한 전사인자 NF-κB의 전이조절

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아그마틴은 L-arginine의 탈탄소반응에 의해 형성된 일차아민으로서 동물의 뇌에서 합성되어지는 내재성 clonidine 대체 물질이다. 본 연구에서는 허혈손상을 받은 별아교세포에서의 아그마틴에 의한 보호효과 조사하고 그 기전을 탐색해 보았다. 아그마틴의 보호효과는 그 작용시점에 따라서 초기에는 괴사를 감소시키고 손상이 끝날 시점에는 예정세포사를 억제하여 세포보호효과를 나타냄을 확인하였다. 아그마틴은 NF-κB의 핵으로의 전이를 증가하는 것을 관찰하였으며, IKKα/β를 활성화 시킴으로써 IκBα를 통해 NF-κB를 조절하는 것을 확인하였다. 또한 아그마틴이 ERK의 활성화를 증가시키고 JNK의 활성화를 감소시키는 것을 관찰하였으며, p38의 발현을 조절하지는 못하는 것으로 확인하였다. NF-κB, ERK 그리고 JNK의 저해제를 사용하여 세포사멸정도를 확인한 결과, 아그마틴

은 NF-κB, ERK 그리고 JNK의 신호전달 조절을 통하여 별아교세포의 세포사를 억제시킴을 확인하였다. 아그마틴에 의해 조절되는 NF-κB 활성화와 MAPK의 연관성을 좀더 자세하게 조사한 결과, ERK 저해제를 사용한 경우 아그마틴에 의해 증가된 p-IκBα 활성화가 억제되었으나, NF-κB 저해제와 아그마틴을 함께 처치한 경우에는 p-IκBα의 발현이 증가한 것을 관찰할 수 있었다. JNK 저해제를 사용한 경우에도 아그마틴에 의해 증가된 p-IκBα 활성화가 억제되었으며, 아그마틴을 함께 처치한 경우에는 p-IκBα의 발현이 더 심하게 억제되었다. 따라서 아그마틴이 ERK와 JNK 신호전달을 조절하지만, 아그마틴에 의한 NF-κB의 활성화기전은 ERK 신호전달과는 연관되어져 있지 않는 것으로 생각되며, JNK 신호전달과는 무관하지 않음을 확인할 수 있었다. 특히 아그마틴에 의한 NF-κB의 활성화가 JNK의 활성을 조절하는 것으로 생각되었다. 또한 아그마틴에 의한 NF-κB의 활성화 조절은 Bcl-2의 발현을 증가시킴으로써 별아교세포의 세포사를 억제하는 것으로 생각되었다. 별아교세포에서의 아그마틴의 보호작용과 관련된 신호전달기전의 이해를 통해서, 궁극적으로 뇌허혈 손상을 막는 치료제 개발을 위한 기초가 될 것이다.

핵심되는 말 : 아그마틴, 허혈 손상, 별아교세포, NF-κB 전이, MAPK, Bcl-2