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Agmatine Attenuates Nitric Oxide Synthesis and Protects ER-structure from Global Cerebral Ischemia in Rats

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ABSTRACT In ischemic strokes, apoptosis is caused by excitotoxicity, ionic imbalance, oxidative/nitrosative stress, and apoptotic-like pathways. Nitric oxide (NO), a free radical, is elevated after ischemic insult. NO, which is generated primarily by neuronal nitric oxide synthase (nNOS) and inducible nitric oxide synthase (iNOS), promotes neuronal damage following ischemia. Evidence obtained in recent years has demonstrated that endoplasmic reticulum (ER)-mediated cell death plays an important role in cerebral ischemia. Agmatine is an endogenous substance synthesized from L-arginine by arginine decarboxylase (ADC) and is present in mammalian brain. We had previously reported that agmatine contributes to neuroprotection against ischemic injury. In continuation of our earlier work, we intended to investigate whether agmatine protects brain from transient global ischemia, and also tried to determine the neuroprotective mechanism of agmatine. Twenty minutes of transient global ischemia was induced by 4 vessel occlusion (4-VO). Agmatine (100 mg/kg, IP) was administered simultaneously with reperfusion. Samplings of brain were done at 6, 24, 48, and 72 h after reperfusion to determine the effect of agmatine on ischemic injured hippocampus. ER-damage was also investigated using electron microscope. Results showed that agmatine treatment prevented delayed neuronal cell death in hippocampal CA1 neurons after global cerebral ischemia. It also blocked NOS expression in the rat brain. Agmatine induced the increased expression of glucose-regulated protein 78 (Grp78). These results suggest that agmatine inhibits the production of NO by decreasing the expression of nNOS and iNOS on global forebrain ischemia and the neuroprotective effect of agmatine were concerned with the ER stress-mediated condition.

Key words : Agmatine, Global ischemia, Nitric oxide synthase, Neuronal cell death, ER stress

INTRODUCTION

Ischemic stroke is the third leading cause of death in industrially advanced countries and a major cause of long-lasting disability (Lo *et al.*, 2003). Although a large number of compounds have been proven to reduce ischemic injury, clinical trials have been unsuccessful because of their toxic side effects. Thus, the development of new drugs and discovery of novel mechanisms for treating cerebral ischemia are very much needed (Lipton, 1999). In ischemic strokes, apoptosis is caused by excitotoxicity, ionic imbalance, oxidative/nitrosative stress, and apoptotic-like pathways (Zhang *et al.*, 2004). Increase in calcium ions mediated by glutamate receptors causes an ionic imbalance and the production of reactive oxygen families, which directly impair lipids, proteins, nucleic acids, and secrete apoptotic proteins (Love, 1999; Droge, 2002; Lo *et al.*, 2003).

A main event during ischemia is the generation of free radicals, which provoke damage to lipids, DNA, and proteins and cause neuronal death (Moro *et al.*, 2004). They also contribute to the breakdown of the blood-brain barrier and brain edema (Moro *et al.*, 2004). Nitric oxide (NO) is one of these radicals and is elevated after ischemic insult (Love, 1999; Paul, 2004). NO is generated primarily by neuronal and inducible NO synthases (NOS) and promotes neuronal damage followed by ische-

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mia (Love, 1999; Moro *et al.*, 2004; Paul, 2004). Ischemia-induced NO overproduction is caused in part by glutamatergic-mediated increase in intracellular calcium concentration, which results in calmodulin-dependent upregulation of NOS (Moro *et al.*, 2004; Paul, 2004). NO is enzymatically synthesized from L-arginine and is massively increased by ischemia (Sastre *et al.*, 1996). Three NOS have been reported (eNOS, nNOS, and iNOS), were named according to their originally defined endothelial (eNOS) and neuronal (nNOS) localizations, or ability to be upregluated when induced (iNOS) (Moro *et al.*, 2004). NO has also been shown to inhibit mitochondrial respiration *via* competition with oxygen for cytochrome oxidase and plays an important role in the initiation of apoptosis (Horn & Limburg, 2001; Nomura, 2004). Although little has been reported on the effects of NO which would warrant them as a subject of clinical investigation, there is no doubt that NO plays a pivotal role in mediating oxidative stress (Warner *et al.*, 2004).

The endoplasmic reticulum (ER) is the site of protein synthesis and protein folding. Perturbations of ER homeostasis affect protein folding and cause ER stress (Paschen, 2003). ER can sense stress and respond to it through translational attenuation, upregulation of genes for ER chaperones and related proteins, and the degradation of unfolded proteins by a quality-control system (Qi *et al.*, 2004b). Evidence obtained in recent years has demonstrated that ER-mediated cell death plays an important role in cerebral ischemia (Qi *et al.*, 2004a). Therefore, targeting the ER may provide a therapeutic approach for blocking the pathological process induced by cerebral ischemia (Hayashi *et al.*, 2004). However, no pharmacological approaches for treating ischemia-induced ER dysfunction have been reported.

Agmatine, [$(NH_2(CH_2)_4NH_2C(NH=)NH$] is an endogenous substance synthesized from L-arginine by arginine decarboxylase (ADC) (Tabor & Tabor, 1984) and is present in mammalian brains (Li *et al.*, 1994; Raasch *et al.*, 1995). Previous studies have shown that agmatine may be neuroprotective both *in vitro* and *in vivo* ischemia models (Olmos *et al.*, 1999; Wei *et al.*, 2002). Agmatine is not a precursor for NOS, but is a rather weak competitive inhibitor of various NOS isozymes (Auguet *et al.*, 1995; Galea *et al.*, 1996). NOS generates NO by sequential oxidation of the guanidinium group in L-arginine, and agmatine is an L-arginine analogue with a guanidinium group (Mayer *et al.*, 1989; Gerova & Torok, 2004). This suggests that agmatine may protect the brain from ischemic injury by interfering with NO signaling. Also, we have previously studied the effects of agmatine on the middle cerebral artery occlusion (MCAO) model in mice and reported that agmatine contribute to neuroprotection against ischemic injury (Kim *et al.*, 2004).

The typical feature of global ischemia is a brief or intermediate occlusion followed by recirculation and long-term recovery (Kim *et al.*, 2001; Fukunaga & Kawano, 2003; Katsuta *et al.*, 2003). Global ischemia, as occurs in cardiac arrest, can only be sustained upto a period of 12 minutes (Siesjo *et al.*, 1995). Transient global cerebral ischemia leads to cell death in hippocampal CA1 pyramidal neurons starting 2～3 days after reperfusion (Kohno *et al.*, 1997; Gillardon *et al.*, 1999). This phenomenon is commonly referred to as selective neuronal vulnerability of delayed neuronal cell death. Several mechanisms, glutamate excitotoxicity, calcium overload, free radical injury, and disturbances in protein synthesis, have been implicated in ischemia-mediated neuronal cell death and transcriptional activation of various genes has been demonstrated in postischemic neurons (Iadecola *et al.*, 1995; Lee *et al.*, 2001; Calderone *et al.*, 2003).

In this study, we investigated the effects of agmatine and its NO regulation. We elucidated ER-damage according to the reperfusion time on neuronal death of transient global cerebral ischemia in rats.

MATERIALS AND METHODS

1. Animals

Male Sprague Dawley rats (300～350 g) from Sam tako, INC. (Osan, Korea) were used for present study. All animal procedures were carried out according to a protocol approved by the Yonsei University Animal Care and Use Committee, which was in accordance with the NIH guidelines.

2. Global cerebral ischemia

The animals were anesthetized with 3% isoflurane and maintained during surgery at a level of 2% isoflurane in 70% N_2O and 30% O_2 with spontaneous breathing. Twenty minutes of transient global ischemia was induced by 4 vessel occlusion (4-VO) using the method described originally by Pulsinelli *et al.* (1979). In brief, after the animals were positioned in stereotaxic ear bars (Kopf, Tujunga, CA) with the head tilted down at ～30 to the horizontal, an incision of 1 cm in length was made behind the occipital bone directly overlying the first two cervical vertebrae. The paraspinal muscles were separated from the midline, and the right and left alar foramina of the first cervical vertebrae were exposed using an operating microscope. A 0.5-mm diameter electrocautery needle was inserted through each alar foramen and both vertebral arteries were electrocauterized and permanently occluded. Next, both common carotid arteries were isolated via a ventral, midline cervical incision. An atraumatic arterial clasp was loosely placed around each

common carotid artery without interrupting the carotid blood flow and the incision was closed with a single suture. On the following day, 20 min of 4-VO ischemia was induced by tightening the clamp around the common carotid arteries. Carotid clamps were then removed. Sham-operated animals that underwent surgery were used for non-ischemic controls.

Rectal temperature was controlled at 37±0.5˚C during surgery with a feedback-regulated heating blanket (Homeothermic Blanket Control Unit, Harvard apparatus; Edenbridge, UK). The femoral artery was exposed and catheterized with a PE-50 catheter to allow continuous recording of arterial blood pressure and withdrawal of blood samples for blood gas analysis. After arterial blood pressure was recovered, arterial blood was collected for blood gas analysis.

3. Agmatine administration

Agmatine was purchased from Sigma (St. Louis, MO, USA) and dissolved in normal saline. It was administered at a dose of 100 mg/kg of weight intraperitoneally with reperfusion (Kim *et al.*, 2004). Control animals received normal saline in equivalent volume.

4. Histological assessment of ischemic injury

Histological and immunohistochemical studies were conducted at 6, 24, 48, and 72 hours (each, n=5) after reperfusion. Animals were perfused transcardially with 200 mL of saline followed by 300 mL of 4% paraformaldehyde solution and were decapitated. Brains were removed and postfixed in the same paraformaldehyde solution for days and then embedded in paraffin. Five μm sections were cut through the dorsal hippocampus (anteroposterior coordinate, bregma-3.0 mm) on a microtome. Brain sections were stained with hematoxylin and eosin (H & E). Neuronal counts in a predesignated region of CA1 were obtained from four to five animals per condition.

5. Western blot analysis

For western blot analysis, animals were sacrificed at 6, 24, 48, and 72 hours (each, n=4) after being exposed to global ischemia. Brains were perfused through the heart aorta with cold PBS to rinse out blood, and protein was extracted, as previously described (Lee *et al.*, 2001). The hippocampus region from the rat brain was dissected, homogenized to extract cellular proteins with lysis buffer containing 1× PBS, 1% nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, proteinase inhibitor (PMSF, aprotinin and sodium orthovanadate) and centrifuged at 13,000 rpm for 17 min at 4˚C. The protein concentration in the supernatant was determined by the BCA method (PIERCE, Rockford, USA).

Table 1. Antibodies used for immunohistochemistry and Western blot

Protein	Format	Concentration Immuno/WB	Manufacturer
nNOS	Rabbit polyclonal	1 : 200/−	Upstate[a]
iNOS	Rabbit polyclonal	1 : 5000/−	Chemicon[b]
eNOS	Rabbit polyclonal	1 : 500/−	Transduction Lab[c]
Nitrotyrosine	Rabbit purified IgG	1 : 200/−	Upstate[a]
Hsp70	Mouse monoclonal	−/1 : 1000	Stressgen[d]
actin	Mouse monoclonal	−/1 : 1000	Santa Cruz[e]
Grp78	Rabbit polyclonal	−/1 : 1000	Santa Cruz
GADD153	Mouse monoclonal	−/1 : 2000	Santa Cruz
Bcl-2	Rabbit polyclonal	−/1 : 1000	Santa Cruz

Immuno, Immunohistochemistry; WB, Western blot

[a]Lake Placid, NY; [b]Temecula, CA; [c]Hercules, CA; [d]Victoria, Canada; [e]Santa Cruz, CA

Western blot analysis was performed using primary antibodies (Table 1). Equal amounts of protein, 40 μg per condition, were separated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and electrotransferred onto Immobilon-NC membranes (Milipore, MA), which were probed with primary antibodies at 4˚C overnight and secondary antibodies at room temperature for 1 hour. To reduce nonspecific antibody bindings, the membranes were blocked with 5% nonfat dry milk for 1 hour at room temperature before being incubated with primary antibodies. Immunoreactive signals were visualized by chemiluminescence using the ECL detection system (Amersham Life Science, Buckinghamshire, UK) with Kodak X-AR film. Immunoblot signals were quantified using a computer program (GelScopeTM, Imageline INC, CA, USA) (Lee *et al.*, 2001).

6. Immunohistochemistry

Brain sections were fixed with 4% paraformaldehyde and rehydrated. Sections were immunostained with primary antibodies followed by an appropriate biotinylated secondary antibody. Stains were visualized using the ABC kit (Vector Laboratories Inc, CA, USA) (Lee *et al.*, 2001), then reacted with diaminobenzidine (DAB) as a substrate (Sigma, St. Louis, USA) and counterstained with hematoxylin. Results were recorded with a DP-70 digital camera (Olympus, Japan).

7. Transmission electron microscopy

For electron microscopy (EM) studies, animals were sacrificed at 24 hours (each, n=3) after global ischemia. Brains were perfused through the heart aorta with 2.5% glutaraldehyde and 2% paraformaldehyde solution. The brain sections were immersed in a solution containing 3% glutaraldehyde in 0.1 M cacodylate buffer for 1 h, rinsed in cacodylate buffer and postfixed for 1 h in 1% OsO_4 solution in the same buffer. The fixed

tissue blocks were then dehydrated through gradient concentrations of ethanol, transferred to propylene oxide, and specimens were embedded in Epon 812-Araldite. Thin sections for ultrastructural evaluation were cut on an LKB ultramicrotome with a diamond knife, contrasted with uranyl acetate and lead citrate, and examined in a Siemens Elmiskop 102 electron microscope (Siemens, Berlin, Germany).

RESULTS

1. The effect of agmatine on delayed neuronal death after transient global ischemia

Ischemic neuronal damage in hippocampal neurons with agmatine treatment following different reperfusion durations in global cerebral ischemia, as evaluated by H & E stanining, was less than that in experimental control groups (Fig. 1A). H & E staining demonstrated that only a few neurons showed ischemic changes at 6 hours of reperfusion after 20 minutes of 4-VO without agmatine treatment (EC6). Almost all neurons showed ischemic changes in the CA1 region in a time-dependent manner and many neurons died in the CA1 subfield at 72 hours of reperfusion after 20 minutes of 4-VO without agmatine treatment (EC72). Whereas neurons of agmatine treatment groups were shaped like normal neurons (Fig. 1A).

2. Long-term effect of agmatine after global ischemia

To investigate the side effects of agmatine, we studied long-

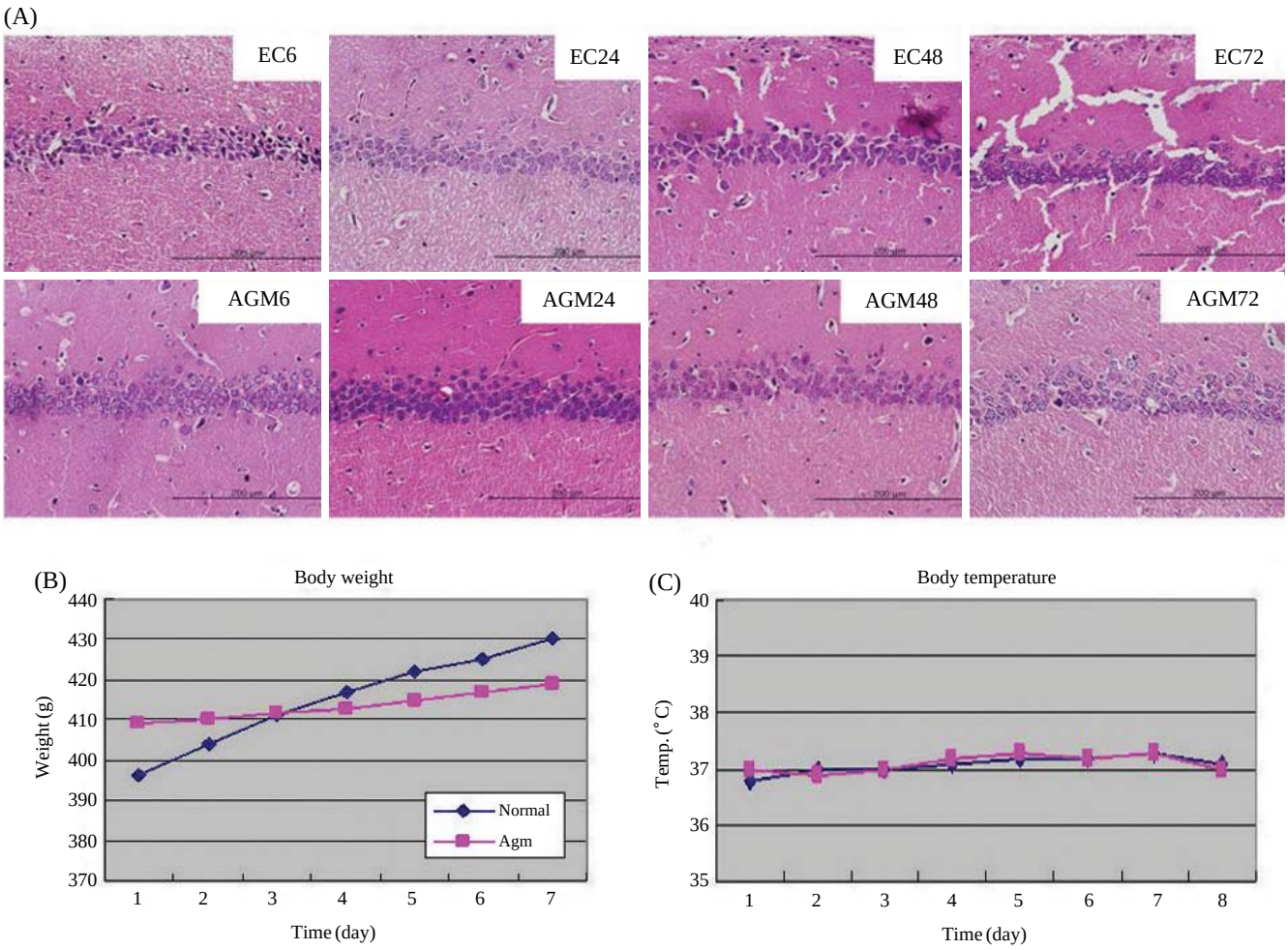

Fig. 1. Neuronal cell death in the rat hippocampus (CA1) was reduced with agmatine treatment following global cerebral ischemia. Hippocampus (CA1) was stained with H & E following different reperfusion durations in global cerebral ischemia with or without agmatine (A). Physiological conditions, body weight (B), and body temperature (C) were not significantly different between normal control and agmatine treatment group following global cerebral ischemia. EC6, 6 hours of reperfusion after 4-VO; EC24, 24 hours of reperfusion after 4-VO; E48, 48 hours of reperfusion after 4-VO; EC72, 72 hours of reperfusion after 4-VO; AGM6, 6 hours of reperfusion after 4-VO with agmatine treatment; AGM24, 24 hours of reperfusion after 4-VO with agmatine treatment; AGM48, 48 hours of reperfusion after 4-VO with agmatine treatment; AGM72, 72 hours of reperfusion after 4-VO with agmatine treatment; Normal, normal control group; Agm, agmatine treatment group. Scale bar is 200 μm.

Table 2. Mortality of 4-vessel model in transient global ischemia

Group	Total animal	survival animal	% of mortality
EC_{10}	11	7	36.4
EC_{20}	11	6	45.5
EC_{30}	9	3	66.7
Agm_{10}	7	5	28.6
Agm_{20}	8	5	37.5
Agm_{30}	5	3	40

EC_{10}, 10 minutes of occlusion; EC_{20}, 20 minutes of occlusion; EC_{30}, 30 minutes of occlusion; Agm_{10}, 10 minutes of occlusion with agmatine treatment; Agm_{20}, 20 minutes of occlusion with agmatine treatment; Agm_{30}, 30 minutes of occlusion with agmatine treatment

term survival changes with and without agmatine treatment. For mortality assessment following different occlusion durations, the animals were sacrificed at 10 minutes, 20 minutes, and 30 minutes of occlusion. However, long-term effects of agmatine was also studied by sacrificing the animals at 24, 72, and 168 hours (each, n=3) after reperfusion.

The animal model with 4-VO showed different mortality rates with or without agmatine treatment (Table 2). The percentages of mortality were recorded on 36.4, 45.5, and 66.7% in 10 minutes of occlusion without agmatine treatment (EC_{10}), 20 minutes of occlusion without agmatine treatment (EC_{20}), and 30 minutes of occlusion without agmatine treatment (EC_{30}) groups, respectively. The percentages of mortality were 28.6, 37.5, and 40% in 10 minutes of occlusion with agmatine treatment (Agm_{10}), 20 minutes of occlusion with agmatine treatment (Agm_{20}), and 30 minutes of occlusion with agmatine treatment (Agm_{30}) groups, respectively. Mortality rate was higher in the experimental control group compared with the agmatine treatment group. Agmatine reduced not only neuronal death induced by the 4-VO model following different occlusion and reperfusion durations, but also ameliorated the animal's survival following different occlusion durations after transient global ischemia.

To study the long-term effects of agmatine, agmatine (100 mg/kg, IP) was injected daily with reperfusion on the first day. Withdrawal of continuous agmatine administration at 24, 72, and 168 hours (each, n=3) showed no remarkable changes as compared to normal controls. Body weights steadily increased in both the normal control (normal) and agmatine treatment (agm) groups (Fig. 1B). Body temperature was always maintained at $37\pm0.5^{\circ}C$ (Fig. 1C). Agmatine did not affect food intake or the body temperature of animals in any groups. It has been proven to exhibit low systemic toxicity.

3. Agmatine has shown the neuroprotective mechanism by suppressing the expression of NOS

The mechanism of neuroprotective effect of agmatine with NO was investigated by histological assessment of three isoforms of NOS, and the formation of peroxynitrite by a reaction with NO.

To study the effect of agmatine on NO, the expression of nNOS, iNOS, and eNOS were examined in the hippocampus of the rat brain after 20 minutes of occlusion in 4-VO global models. Animals were killed by decapitation at 24 and 72 hours (each, n=4) after being exposed to global ischemia.

The number of nNOS positive neurons was increased at 72 hours after 4-VO without agmatine treatment (EC72) and decreased at 72 hours after 4-VO with agmatine treatment (AGM72) in the hippocampal CA1 subregion (Fig. 2A). Similarly, damaged and numerous iNOS-positive neurons were observed in all experimental control groups. The number of iNOS positive cells was effectively decreased in AGM24 (Fig. 2B). In contrast, the number of eNOS positive neurons was unchanged in all groups (data not shown). Intraperitoneal administration of agmatine (100 mg/kg) simultaneously with reperfusion reduced immunoreactivity for nNOS and iNOS expression after ischemia.

4. Blockade of nitrotyrosine production by agmatine after transient global ischemia

To assess the amount of NO production in tissue samples, we examined the immunoreactivity for 3-nitrotyrosine, a marker of peroxynitrite, the reaction product of NO and superoxide.

Global ischemia induced a pronounced increase in nitrotyrosine expression in pyramidal neurons of CA1 in the hippocampus. Nitrotyrosine expression was increased in CA1 neurons at 24 hours after reperfusion, and maintained for 72 hours postischemia in experimental control groups (Fig. 3). In contrast, the increase of nitrotyrosine expression was dramatically suppressed in the agmatine treatment groups, particularly in AGM24 and AGM72 in the hippocampus (Fig. 3).

5. Agmatine protects neurons from transient global ischemia concerned with ER stress-mediated apoptosis

To investigate the effects of agmatine on ER stress, the expression of ER stress-mediated proteins, heat shock protein 70 (Hsp70), glucose-regulated protein 78 (Grp78), C/EBP homologous protein (CHOP), and B-cell CLL/lymphoma 2 (Bcl-2), were examined in the hippocampus and cerebral cortexes of rat brains after global ischemia by immunoblotting. The level of Hsp70 was increased and maintained high levels in all groups (Fig. 4). The level of Grp78 in the Agmatine treatment group after 4-VO global ischemia was higher than experimental control groups, especially at 6 hours (AGM6) and 24 hours (AGM24) of reperfusion after 4-VO with agmatine treat-

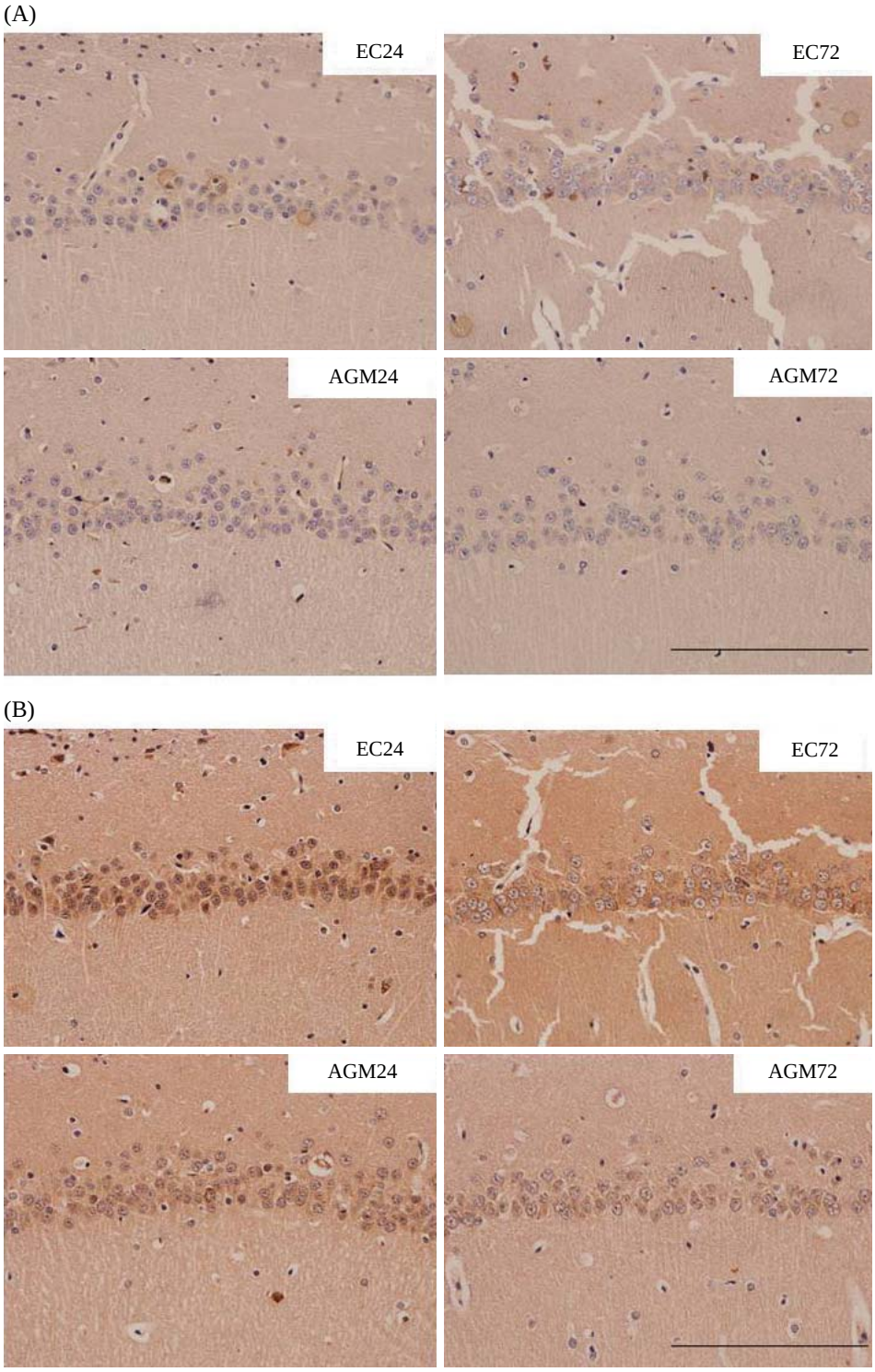

Fig. 2. The expressions of nNOS and iNOS in the rat hippocampus (CA1) after global cerebral ischemia with or without agmatine. The number of nNOS positive neurons in CA1 of the hippocampus was decreased in AGM72 in immunohistochemistry (A). The number of iNOS positive neurons in CA1 of the hippocampus was also significantly decreased in AGM24 in immunohistochemistry (B). EC24, 24 hours of reperfusion after 4-VO; EC72, 72 hours of reperfusion after 4-VO; AGM24, 24 hours of reperfusion after 4-VO with agmatine treatment; AGM72, 72 hours of reperfusion after 4-VO with agmatine treatment. Scale bar is 200 μm.

ment (Fig. 4). The level of CHOP expression was more in the agmatine treatment group (Fig. 4). Bcl-2 level was higher in no agmatine treatment group (EC48) compared with agmatine treatment group (AGM48) at 48 hours of reperfusion after 4-VO.

6. Electron microscopic study mediated by ER stress for transient global ischemia

Electron microscopy of normal control revealed the presence of vivid chromatin, distinct nuclear membrane, strong ER structures, and Golgi apparatus (Fig. 5a, d, and g).

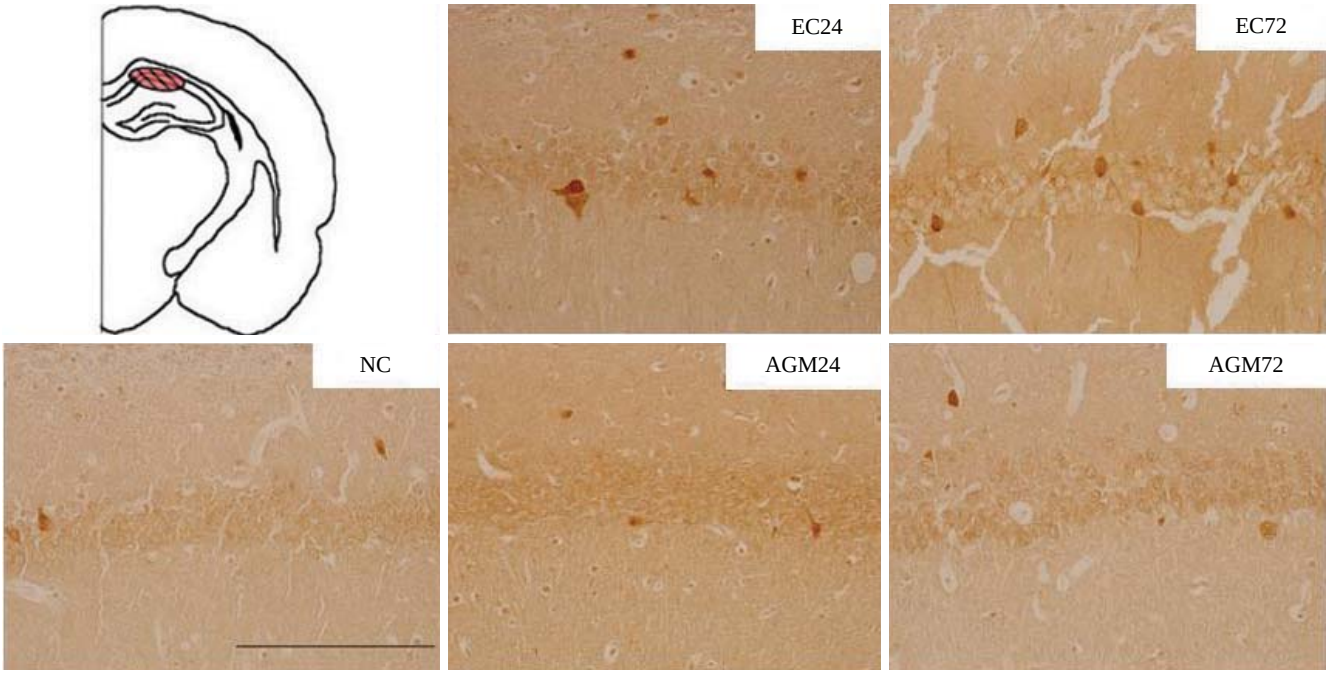

Fig. 3. Immunohistochemistry of nitrotyrosine in postischemic CA1 neuron of rat hippocampus following the reperfusion time course. Nitrotyrosine expression was significantly decreased by agmatine treatment in the AGM24 and AGM72 groups. NC, normal control group; EC24, 24 hours of reperfusion after 4-VO; EC72, 72 hours of reperfusion after 4-VO; AGM24, 24 hours of reperfusion after 4-VO with agmatine treatment; AGM72, 72 hours of reperfuion after 4-VO with agmatine treatment. Scale bar is 200 μm.

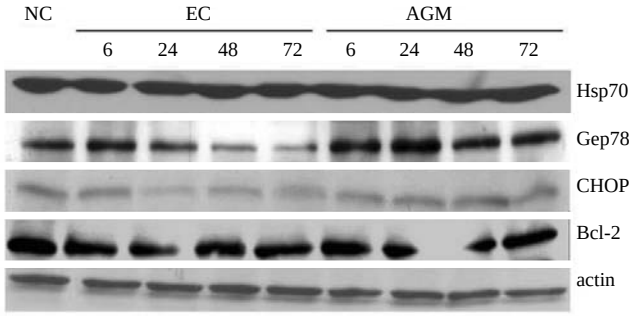

Fig. 4. The expressions of Hsp70, Grp78, CHOP and Bcl-2 in the rat hippocampus following global cerebral ischemia using Western blot analysis. The level of Grp78 was higher in AGM than EC from 6 hours of reperfusion to 72 hours after 4-VO. NC, normal control group; EC, experimental control group; AGM, agmatine treatment group; Hsp70, heat shock protein 70; Grp78, glucose-regulated protein 78; CHOP, C/EBP homologous protein; Bcl-2, B-cell CLL/lymphoma 2.

Our EM study indicated significant morphological changes in the ER and mitochondria in the experimental control group (EC), with typical apoptotic patterns of compaction and segregation of chromatin, destruction of ER lumen, and disruption of mitochondria. After 24 hr of reperfusion without agmatine, swollen rough ER were prevalent (Fig. 5b, e, and h). Swollen rough ER and free ribosomes that were released from the ER found to be increased in EC, whereas the mitochondria and ER lumen remained morphologically normal at 24 h of agmatine treatment. However, most of the cells in the agmatine-treated rat brain showed no significant morphological differences (Fig. 5c, f, and i).

DISCUSSION

In the present study, we used rat as a model for transient global ischemia. Twenty minutes of ischemia resulted in marked damage to the hippocampus as determined by H & E staining (Lee *et al.*, 2001). On the other hand, we demonsrated that intraperitoneal administration of agmatine protected the brain from ischemic injury. Simultaneously, we found that agmatine can effectively suppress ER dysfunction *in vivo*. Moreover, the present findings indicate that the protective effect of agmatine on ischemic injury may be mediated in part by restoration of ER dysfunction.

In cerebral ischemia, apoptosis is caused by excitotoxicity, ionic imbalance, oxidative/nitrosative stress, and apoptotic-like pathways (Love, 1999; Yuan & Yankner, 2000; Droge, 2002; Lo *et al.*, 2003). In correlation with these events, there is an significant increase in the generation of NO, resulting from activation of the NOS on both resident and infiltrating cells (Blantz *et al.*, 2000). Excessive generation of NO is thought to

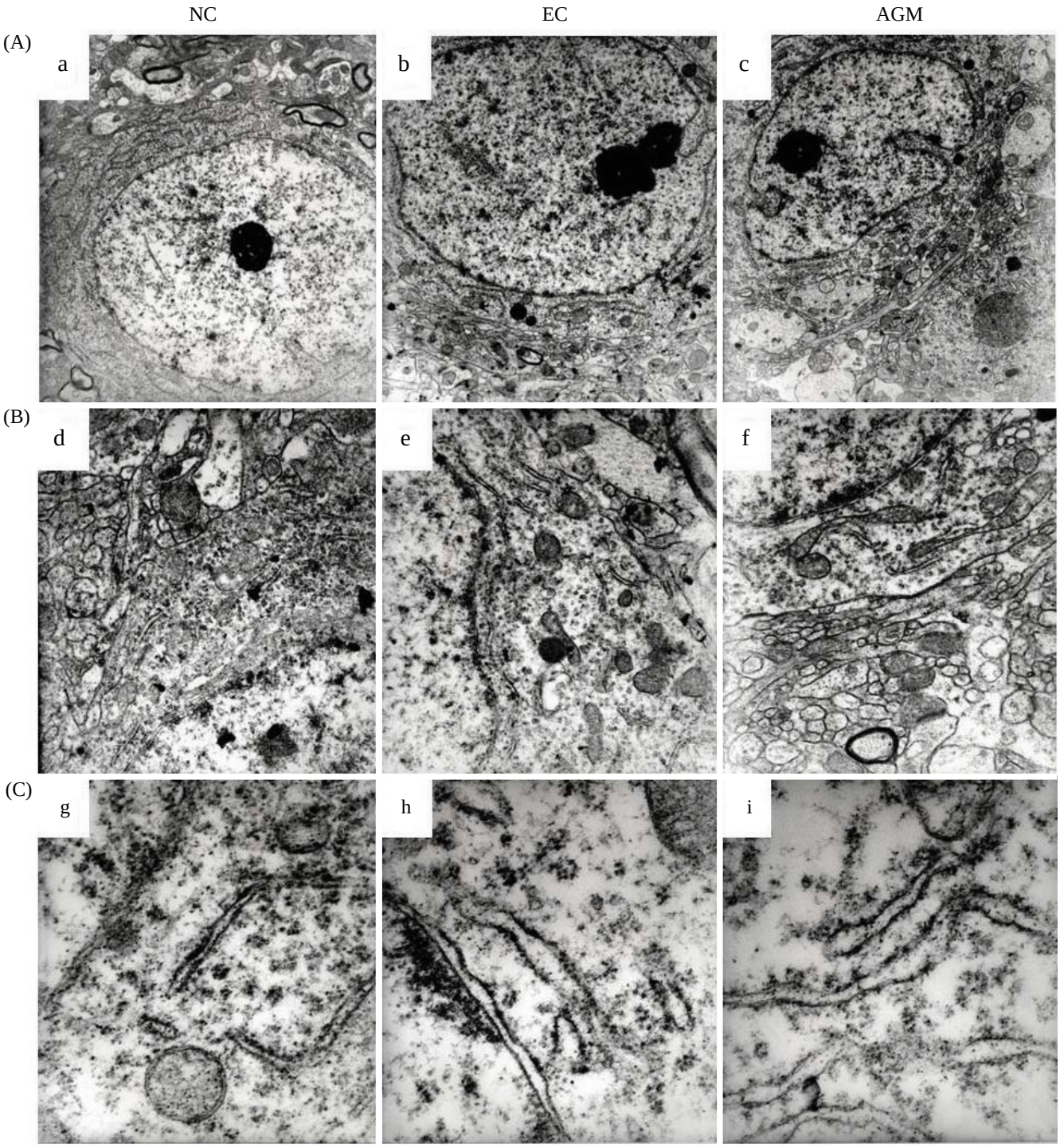

Fig. 5. Electron microscopy of rat hippocampal cell injured by ER stress at 24 hours of reperfusion after 4-VO. Electron micrographs show ultrastructural alterations in individual neurons in the hippocampus. The control hippocampal neuron had a large nucleus, relatively small cytoplasm, and intact mitochondria, ribosomes on the ER lumen and other organelles. ER structure was severely destroyed and several ribosomes were innervated in the cytoplasm of EC. In contrast, the ER structure of AGM was shown intact, like NC. (A), ×15960; (B), ×37200; (C), ×124800 original magnification. NC, normal control group; EC, experimental control group; AGM, agmatine treatment group.

play an important role in ischemic injury (Raghavan & Dikshit, 2004). However, exogenous and endogenous radical scavengers have been demonstrated to suppress nitrosative stress and thus prevent ischemic injury (Tabor & Tabor, 1984; Siesjo *et*

al., 1995; Kohno *et al.*, 1997). Agmatine has been shown to exert a protective effect against cerebral ischemic injury by blocking NMDA receptor activation and inhibiting NOS activity (Olmos *et al.*, 1999; Yang & Reis, 1999). However, ample evidence suggested that ER damage is involved in neuronal cell death induced by cerebral ischemia (Hori *et al.*, 2002; Nomura, 2004; Xu *et al.*, 2004). Basing on the available reports, we investigated the effect of agmatine on ER dysfunction under pathological conditions.

In the 4-VO global model, we observed a significant decrease in the number of nNOS and iNOS positive cells in agmatine treatment group 24 hr after 20 minutes occlusion. We already demonstrated in our previous report, agmatine decreased infract sizes and appeared to reduce nNOS expression to a greater extent than iNOS in the MCAO mouse model (Kim *et al.*, 2004). There are at least three likely mechanisms for agmatine neuroprotective activity. Agmatine has been shown to reduce excitotoxicity *in vitro* by blocking NMDA receptor activiation (Olmos *et al.*, 1999; Yang & Reis, 1999). Agmatine, an α-2 adrenoceptor agonist, has been shown to protect neurons from injury *in vivo* and *in vitro* (Li *et al.*, 1994; Wei *et al.*, 2002). Lastly, an NOS antagonist and generation of NO have been implicated in ischemic brain injury (Iadecola, 1997).

Agmatine also has been shown to be a competitive inhibitor of both nNOS and iNOS (Regunathan & Piletz, 2003; Kim *et al.*, 2004). In our present study, we found that the induction of nNOS and iNOS by global ischemia was inhibited with the administration of agmatine. Agmatine also effectively reduced the production of nitrotyrosine in the hippocampus after global ischemia.

An important source of oxidative stress-mediated brain damage is the oxidant reactions caused by the formation of peroxynitrite, a powerful oxidant that results from the interaction between NO and superoxide. This anion has been shown to cause cell damage by several mechanisms, including lipid peroxidation, tyrosine nitration, sulfhydryl oxidation and nitrosylation, DNA breakage, etc (Kontos, 2001; Fukunaga & Kawano, 2003).

In contrast to the toxic effects of nNOS and iNOS, eNOS mediates vasodilation, inhibites the platelet aggregation and leukocyte adhesion to the endothelium, which are the beneficial effects that would result in protection following cerebral ischemia (Moro *et al.*, 2004; Paul, 2004; Raghavan & Dikshit, 2004). In our findings, there were no significant changes in the number of eNOS positive cells in immunoreactivity.

Our data showed a weak expression in the level of the transcription factor CHOP and inactivation of Grp78 in the ischemic hippocampus in rats that underwent twenty minutes of global ischemia followed by a given period of recovery. The obtained results indicate that global ischemia caused severe ER damage and triggered ER stress-associated apoptosis. On the other hand, agmatine treatment slightly increased the level of CHOP and strongly activated Grp78 in the hippocampus supporting neuroprotective activity. According to our knowledge, there have been no reports on the neuroprotective mechanism of agmatine related to ER stress till now.

ER stress response activates two transcription factors, CHOP and NF-kB, whose role in the stress response has not been fully elucidated (Qi *et al.*, 2004b). C/EBP homologous protein (CHOP), also known as Growth Arrest and DNA Damage-inducible gene 153 (GADD153) (Qi *et al.*, 2004b), was first isolated and screened to identify genes responsive to UV treatment, but subsequent studies have recognized this transcription factor to be the most sensitive to alterations in the ER culminating in unfolded protein response (UPR) (Oyadomari & Mori, 2004). Expression of GADD153 is activated by all agents that up-regulate ER chaperons, and like chaperones, it is induced by activating transcription factor 6 (ATF6) and activating transcription factor 4 (ATF4) (Oyadomari & Mori, 2004). Several studies have shown the role of GADD153 in negative regulation of cell growth and differentiation, and it has also been well implicated in apoptosis induction (Renata & Alan, 2003). Our study indicated that GADD153 sensitizes cells to ER stress by down-regulation of Bcl-2 family and by depletion of glutathione (Kadowaki *et al.*, 2004; Oyadomari & Mori, 2004). However, it is largely unknown and further investigations are to be done that how the changes in expression of these target genes relate to apoptosis in ischemic injury.

Glucose-regulated protein (Grp) 78 is a molecular chaperone involved in the UPR, termed the unfolded protein response in the ER of all eukaryotic cells (Xu *et al.*, 2004). The ER chaperone Grp78 binds to the ER luminal domains in both PERK and IRE1, which serves to repress activation of their cytosolic catalytic domains (Kumar *et al.*, 2003). GRP78, the major sensor of ER stress, seems to be a promising target in neuroprotection and cancer therapy, and a well known marker of tumor progression. Modification of ER stress response can help neurons withstand the stressful conditions during cerebral ischemia (Banhegyi *et al.*, 2007). The increase in Grp78 provides significant cytoprotection against toxic agents, including thapsigargin (Reddy *et al.*, 2003).

Bcl-2 is one among several key regulators of apoptosis, which are essential for proper development, tissue homeostasis, and protection against foreign pathogens (Paschen, 2003). The anti-apoptotic function of Bcl-2 can also be regulated through proteolytic processing and phosphorylation. Bcl-2 may promote cell survival by interfering with activation of the cyto-

chrome c/Apaf-1 pathway through stabilization of the mitochondrial membrane (Paschen *et al.*, 2003; Verkhratsky & Toescu, 2003).

We demonstrated the neuroprotective effects of agmatine administered simultaneously following reperfusion in global cerebral ischemia by performimg western blot analysis and immunohistochemistry. Agmaine has numerous possible pathways to inhibit neuronal cell death, but mainly by blocking the NMDA channel and competitive inhibition of NOS. We focused on the relation of agmatine to NOS and NO. In addition, an investigation was done on the possible protective effects of agmatine against ER dysfunction related NO. Our results indicated that agmatine significantly inhibited ER stress-mediated apoptotic signals induced by transient global ischemia and attenuated ER stress in global ischemia.

Agmatine has recently been shown to inhibit NOS in isolated rat aorta (Auguet *et al.*, 1995) and in rat brains (Li *et al.*, 1994; Li *et al.*, 1995). In fact, the inhibitory effect of agmatine on NO synthesis has been implicated in its inhibitory effect on morphine abstinence syndrome and in its role as antidepressant (Auguet *et al.*, 1995; Galea *et al.*, 1996). Agmatine can also antagonize N-methyl-D-aspartate (NMDA) receptor-induced effects in hippocampal neurons (Olmos *et al.*, 1999; Yang & Reis, 1999). Apart from these neuromodulatory roles, agmatine is a metabolic precursor of polyamines, which by themselves induce various central effects (Lo *et al.*, 2003).

It is well established that the rat transient global ischemia model mimics the condition after transient cardiac arrest and causes selective neuronal death in vulnerable regions, such as hippocampal CA1 pyramidal cells, Purkinje cells of the cerebellum, and neurons in the third to fifth layers of the cerebral cortex (Kim *et al.*, 2001; Katsuta *et al.*, 2003). This vulnerability has been attributed to numerous factors, such as glutamate neurotoxicity, calcium accumulation, expression of cell suicide genes, activation of apoptotic proteins, and mitochondrial dysfunction (Siesjo *et al.*, 1995). Global cerebral ischemia triggers activation of apoptotic pathways in neurons destined to die (Kohno *et al.*, 1997; Sugino *et al.*, 2000). It has been emphasized on the critical role of mitochondrial dysfunction in these mechanisms after recirculation.

The major finding in our current investigation is that agmatine protects brain tissue against ischemic injury (Ketteler *et al.*, 1994; Reyes *et al.*, 1994). This effect appears to be mediated through inhibiting the expression of nNOS and iNOS and by inducing the over-expression of Grp78 associated with ER stress in ischemic insults.

In conclusion, agmatine may be a promising therapeutic substance for treatment of cerebral ischemia. Considering the findings of the present study, we speculate that agmatine has multiple effects, although the mechanism of its actions needs to be elucidated further.

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