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Endogenous Agmatine Induced by Ischemic Preconditioning Regulates Ischemic Tolerance Following Cerebral Ischemia

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Ischemic preconditioning (IP) is one of the most important endogenous mechanisms that protect the cells against ischemia-reperfusion (I/R) injury. However, the exact molecular mechanisms remain unclear. In this study, we showed that changes in the level of agmatine were correlated with ischemic tolerance. Changes in brain edema, infarct volume, level of agmatine, and expression of arginine decarboxylase (ADC) and nitric oxide synthases (NOS; inducible NOS [iNOS] and neural NOS [nNOS]) were analyzed during I/R injury with or without IP in the rat brain. After cerebral ischemia, brain edema and infarct volume were significantly reduced in the IP group. The level of agmatine was increased before and during ischemic injury and remained elevated in the early reperfusion phase in the IP group compared to the experimental control (EC) group. During IP, the level of plasma agmatine was increased in the early phase of IP, but that of liver agmatine was abruptly decreased. However, the level of agmatine was definitely increased in the ipsilateral and contralateral hemisphere of brain during the IP. IP also increased the expression of ADC—the enzyme responsible for the synthesis of endogenous agmatine—before, during, and after ischemic injury. In addition, ischemic injury increased endogenous ADC expression in the EC group. The expression of nNOS was reduced in the I/R injured brain in the IP group. These results suggest that endogenous increased agmatine may be a component of the ischemic tolerance response that is induced by IP. Agmatine may have a pivotal role in endogenous ischemic tolerance.

Key words: agmatine, cerebral ischemia, ischemia-reperfusion injury, ischemic tolerance, neuroprotection, nitric oxide synthase

INTRODUCTION

Ischemic tolerance is the phenomenon whereby ischemic preconditioning (IP) protects against a subsequent episode of lethal ischemia [1]. Endogenous mechanisms of IP lead to increased cellular resistance against ischemia after one or several episodes of transient ischemia. It has been shown that IP protects pyramidal cells in hippocampal CA1 from subsequent lethal ischemia [2]. The neuroprotective mechanism of IP is thought to occur via

numerous signal transduction pathways involved in cell protection. Heat shock proteins, immediate early genes, antioxidant enzymes, and anti-apoptotic pathways provide ischemic tolerance and increased cell survival after ischemia [3-5]. It is also thought to involve endoplasmic reticulum and DNA repair mechanisms [6]. However, the exact molecular mechanisms remain unclear despite the many numbers of studies.

Agmatine, a molecule synthesized via decarboxylation of L-arginine by arginine decarboxylase (ADC), is hydrolyzed to putrescine and urea by agmatinase [7, 8]. Several research groups, including ours, have reported that agmatine, ADC, and agmatinase are found in different regions of the mammalian brain, where they provide neuroprotection against various types of cerebral injury [9]. Agmatine is an endogenous clonidine-displacing substance, an agonist for the α2-adrenergic and imidazoline receptors, and an antagonist for the N-methyl-D-aspartate (NMDA) receptors [9-12]. Recent studies have shown that agmatine may be neuroprotective in trauma and ischemia models [7, 13-19]. Agmatine protects neurons against glutamate toxicity, and this effect is mediated through NMDA receptor blockade, with agmatine interacting at a site located within the NMDA channel pore [20]. Being structurally similar to L-arginine, agmatine is also a competitive nitric oxide synthase (NOS) inhibitor [21, 22]. NOS generate nitric oxide (NO) by subsequent oxidation of the guanidinium group in L-arginine, and agmatine is an L-arginine analog with a guanidinium group. Nitric oxide (NO) generated by NOS is known to trigger inflammation and apoptosis after ischemic stroke [23].

We have previously reported that agmatine exerts neuroprotective functions by reducing the size of ischemic infarctions and preventing cerebral neuron loss by reducing NOS expression after focal or global ischemia [18, 24, 25]. These results suggested that agmatine protects the brain from ischemic injury by interfering with NO signaling. Based on the neuroprotective effects of agmatine in cerebral injuries, particularly cerebral ischemic injuries, it is important to investigate the changes in agmatine level and the role of agmatine in ischemic tolerance following IP for defining the protective mechanism of IP. Therefore, we performed this study to determine the effects of agmatine on ischemic tolerance after transient focal ischemia and to determine the systemic level of agmatine during ischemic injury.

MATERIALS AND METHODS

Experimental animals

Eight-week-old male Sprague-Dawley rats (SAMTAKO, Osan, South Korea) weighing 300±20 g were used for all experiments. Rats were allowed free access to food and water before the experi-

ments. Animals were anesthetized with Zoletil50 (0.6 mg/kg) and rompun (0.4 mg/kg) injected intramuscularly. Before and during any surgery, body temperature was maintained at 36.5~37.5°C. All animal procedures were carried out according to a protocol approved by the International Animal Care and Use Committee (IACUC) of Yonsei University Animal Research Center (YLARC, permission No. 2014-0375) using National Institutes of Health (NIH) guidelines. All of the animals were maintained in a specific pathogen-free facility of the YLARC.

Induction of IP and focal cerebral ischemia

Transient middle cerebral artery (MCA) occlusion was conducted as described before [16]. In the IP group, a 60-min major occlusion was induced 3 days after a 10-min occlusion. In the experimental control (EC) group, a 60-min major occlusion was induced 3 days after the sham operation (Fig. 1A). In detail, a rat was anesthetized and placed in a stereotaxic frame. A craniectomy (3 mm in diameter, 6 mm lateral and 2 mm caudal to bregma) was performed with extreme care over the MCA territory using a trephine. The dura was left intact, and a laser Doppler flowmeter probe was placed on the surface of the ipsilateral cortex and fixed to the periosteum. The probe was connected to a laser flowmeter device (OMEGA FLOW, FLO-C1, Neuroscience, Tokyo, Japan) for continuous monitoring of regional cerebral blood flow (rCBF). The right common carotid artery (CCA), external carotid artery (ECA), and internal carotid artery (ICA) were exposed through a ventral midline incision. A 4-0 monofilament nylon suture with a rounded tip (around 160 μm in diameter) was introduced into the CCA lumen and gently advanced into the ICA until rCBF was reduced to 15~20% of the baseline (recorded by laser Doppler flowmetry). After the desired period of occlusion (10 min or 1 hr), the suture was withdrawn to restore the blood flow (confirmed by the return of rCBF to the baseline level). The wound was sutured, and the rat was allowed to recover from anesthesia before returning to the cage with free access to rat chow and water.

Morphometric measurement of brain edema and infarct volume

Animals were decapitated at 0, 1, 2, 4, 7, or 24 hr after ischemia and the brains were rapidly removed and sectioned coronally with 2-mm intervals. The 2nd, 4th, and 6th sections of six serial slices were incubated for 30 min in a 2% 2,3,5-triphenyltetrazolium chloride (TTC) solution at 37°C and fixed in a 4% paraformaldehyde solution. Using a computerized image analysis system (Image J, version 1.36, NIH, USA), the area of infarction of each section was measured. The volume of infarction in each animal was obtained from the product of slice thickness (2 mm) and the sum of infarction ar-
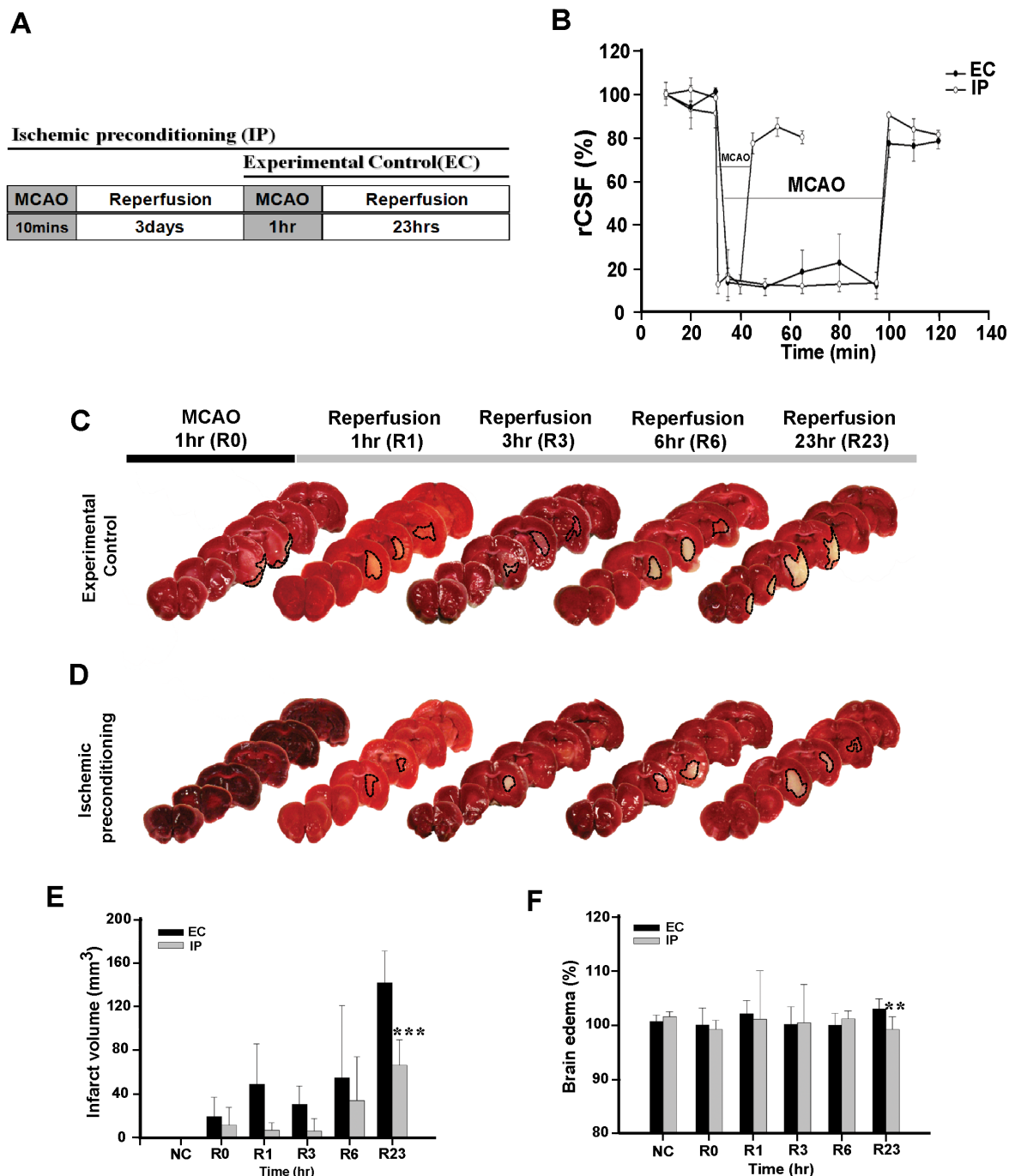
Fig. 1. IP reduced infarct size and brain edema in a rat model of MCAO. (A) Schematics of experimental IP in the rat brain. (B) The rCBF value was not significantly different between EC and IP groups during ischemic injury. Baseline values before MCAO were defined as 100% flow. (C) TTC staining of the ischemic injured brain of EC. (D) TTC staining of the ischemic injured brain with IP. (E and F) IP reduced the infarct volume and brain edema significantly compared to EC at R23. EC, experimental control; IP, ischemic preconditioning; NC, Normal Control; R0, 1 hr after MCAO; R1, 1 hr after reperfusion; R3, 3 hr after reperfusion; R6, 6 hr after reperfusion; R23, 23 hr after reperfusion (**p<0.05 vs. EC; ***p<0.01 vs. EC).

eas in all brain slices examined. Brain edema was determined from the following formula: Brain edema (%)=(the volume of ipsilateral hemisphere/the volume of contralateral hemisphere) ×100 (%).

Agmatine analysis with HPLC

Sample preparation

Brain, liver and plasma samples were prepared according to

the method of Reed and Belleroche with modification [26]. The ipsilateral part of 3rd brain coronal sections, liver, and plasma was quickly stored at -80°C until further use. Agmatine levels were determined by using high-performance liquid chromatography (HPLC) [27]. Briefly, tissue and plasma samples were weighed and homogenized in 0.5 ml of ice-cold 10% (w/v) trichloroacetic acid (TCA) per 150 mg tissue (wet weight) in ice. Sample homogenates were then left on ice for 1 hr and centrifuged at 20,000×g for 25 min. The supernatant was washed five times using an equal volume of ice-cold diethyl-ether, and the aqueous phase was saved. Any remaining ether was evaporated at room temperature for 20 min. A volume of 20 µl of TCA eluted sample and a volume of 20 µl of the OPA-ME derivatizing reagent was mixed for 2 min at room temperature. Next, 20 µl was immediately injected into the HPLC system (Shimadzu, Kyoto, Japan).

Apparatus and chromatographic conditions

The HPLC system consisted of a pump and multi-solvent delivery system (Shimadzu HPLC CLASS-VP, Japan), a RF-10Axl fluorescence detector (the excitation wavelength of 325 nm and the emission wavelength of 425 nm; Shimadzu, Japan), and a Hypersil GOLD 150×2.1, 5-µm column (ThermoFisherScientific). Potassium borate buffer (final concentration 0.2 M, pH 9.4 at 20°C) was prepared by dissolving boric acid in water and adjusting the pH with a saturated solution of potassium hydroxide in a final volume of 250 ml. The buffer was passed through a 0.22-µm filter (Gelman Sciences, MI, USA) and stored at 4°C. The OPA-ME derivatizing reagent was prepared by dissolving 50 mg OPA in 1 ml of methanol and adding 53 µl of ME and 9 ml of 0.2 M potassium borate buffer (pH 9.4); the solution was stored at 4°C for not more than three days before use. Agmatine was measured by derivatizing with OPA-ME. The mobile phase consisted of a mixture of 46% 10 mM potassium dihydrogen phosphate containing 3 mM octylsulfate sodium salt in water (pH 5.93), 34% acetonitrile, and 20% methanol. The mobile phase was filtrated (FHLP04700, 0.45 µm FH, Millipore) and degassed before use.

Quantification and statistical analysis

For the isocratic HPLC method, the concentration of agmatine in each sample was calculated by linear regression analysis of peak heights to the addition of varying concentrations of agmatine to samples [28].

Immunoblotting of ADC

Expression of ADC protein was estimated by immunoblotting in the ipsilateral part of 3rd brain coronal sections and the liver. Immunoblotting was performed using anti-ADC (ATGEN; 1:500,

Gyeonggi-do, South Korea) and anti-actin (Santa Cruz; 1:500, TX, USA) antibodies. Equal amounts of protein (20 µg per condition) were separated on a 10% polyacrylamide gel and electro-transferred onto an Immobilon-P membrane (Millipore, Bedford, MA, USA). Immunoreactive bands were visualized with the ECL detection system (ThermoFisherScientific) using Kodak X-AR film.

Immunohistochemical staining for NOS

For immunohistochemistry, 6-µm 4th brain coronal sections were quickly fixed with 4% paraformaldehyde and embedded in paraffin. Sections were immunostained with antibodies against nNOS (#06-528, Upstate; 1:200, MA, USA) or iNOS (#482728, Calbiochem; 1:200, CA, USA) followed by an appropriate biotinylated secondary antibody. Stains were visualized using the ABC kit (Vector, CA, USA), then reacted with diaminobenzidine (DAB, Sigma, St. Louis. MO, USA). Immunostaining controls were prepared by preparing sections without primary antibodies. All incubation steps were performed in a humidified chamber. The positive area was measured using a computerized image analysis system (Image J v1.36, NIH, USA).

Statistical analysis

Statistical tests to determine differences between groups were performed using the Student's t-test using SPSS 13.0 (IBM, Chicago, IL, USA). A p value<0.05 was considered significant. Data are expressed as the mean±standard deviation (SD), with the exception of HPLC results, for which the mean±standard error of the mean (SEM) is shown.

RESULTS

IP reduced injury after cerebral ischemia

The rCBF pattern measured using laser Doppler flowmetry over the ipsilateral parietal cortex is presented in Fig. 1B. Baseline rCBF recorded before MCA occlusion under steady-state conditions was defined as 100% flow. After MCAO, CBF decreased to 20% in both groups. Ischemia was confirmed when the laser Doppler signal was reduced to 20% of the baseline. rCBF levels were not significantly different between groups. Twenty-four hours after MCAO, infarct volume and edema volume were analyzed. Infarct volume was markedly reduced to approximately 47% in the IP group compared to the EC group at 23 hr of reperfusion following 60-min ischemia (Fig. 1C~E). Moreover, IP reduced the brain edema significantly at 23 hr of reperfusion following 60-min ischemia (Fig. 1F). These results suggest that IP was highly effective in protecting the brain from ischemic injury.

Endogenous agmatine was increased during I/R injury in the IP group

The potential relationship between ischemia and endogenous agmatine levels was determined in the brain using HPLC analysis (Fig. 2). The level of agmatine in normal conditions was 5.65±0.20 μg/mg protein. It was increased after the onset of MCAO more than two-fold to 13.1±1.91 μg/mg protein. After the start of reperfusion, agmatine level was decreased to 9.40±1.51 μg/mg protein at 1 hr of reperfusion and slowly increased to 15.64±1.53 μg/mg protein at 23 hr of reperfusion in the EC group (Fig. 2A). In the IP group, the level of agmatine was found to be significantly increased (15.35±0.63 μg/mg protein) before the onset of MCAO. Moreover, a significantly higher increase (18.92±1.87 μg/mg protein) of agmatine was recorded in the IP group compared to that of the EC group (9.40±1.51 μg/mg protein) at 1 hr of reperfusion. Agmatine level was decreased after 1 hr of reperfusion and reached a plateau from 3 hr of reperfusion to 23 hr of reperfusion in the IP group (8.83±0.44 μg/mg protein, Fig. 2A). However, the level of agmatine in both EC and IP groups was significantly elevated compared to the normal level of agmatine. During the IP period before major I/R injury, the level of agmatine was significantly increased by 10-min MCAO in the brain of the IP group (Fig. 2B). The level of agmatine was significantly increased in both ipsilateral and contralateral hemispheres at 30 min and 1 day after IP. However, the level of agmatine was similar in ipsilateral and contralateral hemispheres 3 days after IP. The level of agmatine in the liver was significantly reduced, showing an opposite tendency to that in the brain during 3 days of IP (Fig. 2C). The level of L-arginine in the
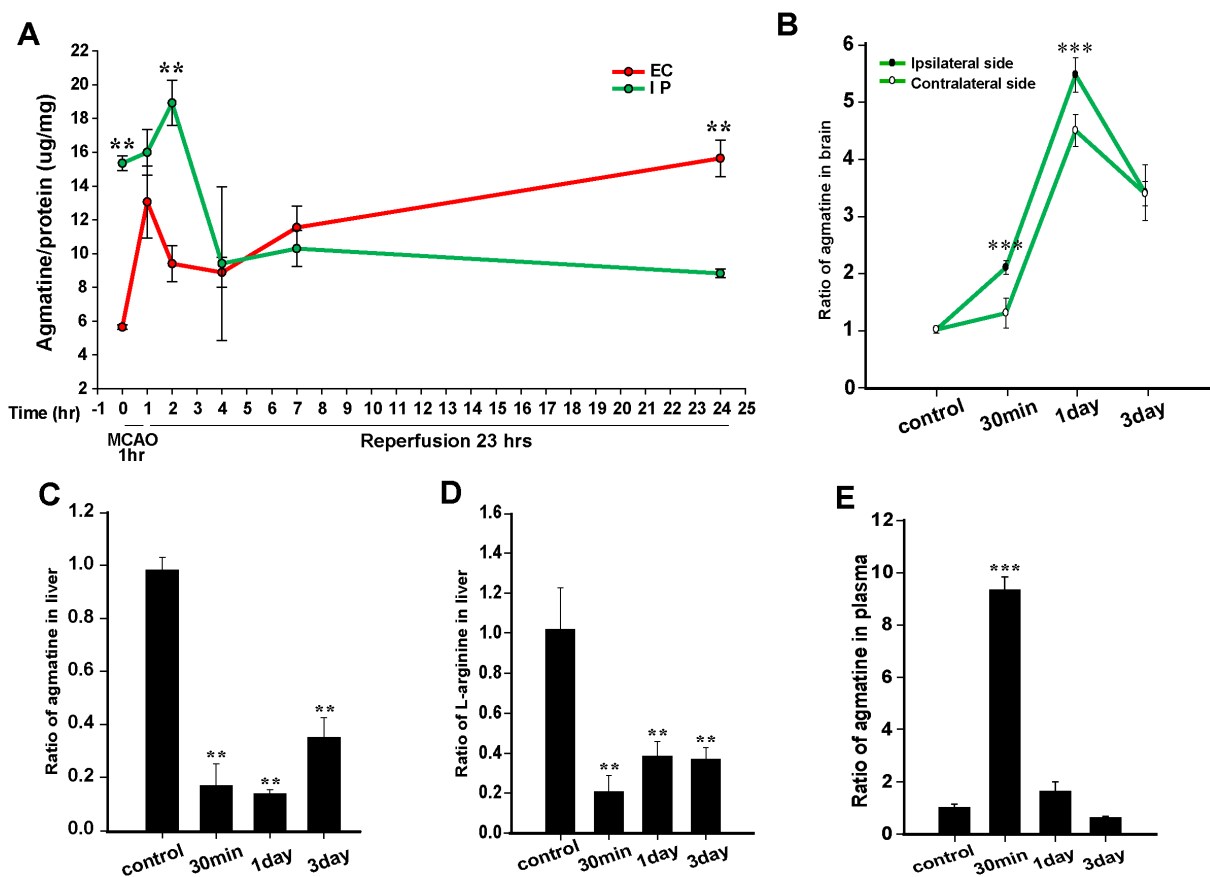

Fig. 2. IP triggers the systemic elevation of agmatine and L-arginine level in rats. (A) The level of agmatine in the rat brain was significantly increased in the IP group during MCAO and at the early reperfusion time. The highest peak was noted in the IP group at 2 hr after injury. The level of agmatine was gradually reduced in the IP group at 23 hr of reperfusion. However, in the EC group, the agmatine level was gradually in the brain at 23 hr of reperfusion. (B) Three days before severe MCAO, experimental animals were subjected to 10-min MCAO. The level of agmatine was significantly increased in the ipsilateral and contralateral hemisphere after 10-min IP compared to normal control (NC). The level of agmatine was still intensified about 3.5 folds at 3 days after IP compared to the normal level of agmatine, but there was no significant difference between ipsilateral and contralateral hemisphere. (C) On the other hand, the level of agmatine in liver was significantly reversed after IP. 30 mins after IP, the level of agmatine was significantly reduced about 20% compared with NC. (D) The level of agmatine precursor, L-arginine, in the liver was significantly suppressed after IP compared with NC. (E) Simultaneously, the level of agmatine in plasma and brain was significantly increased at 30 mins after IP. The ratio of agmatine in the plasma was markedly increased about ten-fold at 30 min after IP (**$p<0.05$ vs. EC; ***$p<0.01$ vs. EC).

liver was also significantly decreased (Fig. 2D). The more significant finding was that plasma agmatine level was significantly increased 30 min after IP, which was synchronized with an increased level of agmatine in the brain (Fig. 2B and E).

ADC, the biosynthetic enzyme for agmatine, was upregulated in the brain and liver by IP

The expression of ADC in the EC and IP groups was significantly upregulated in the ipsilateral brain and liver after cerebral ischemia compared to NC (Fig. 3A) and IP increased the expression of ADC in the liver after cerebral ischemia (Fig. 3B). During the IP period, the level of ADC was higher in the ipsilateral brain and liver in the IP group compared to NC (Fig. 3A). These results indicated that IP led to local ADC upregulation in the brain and systemic ADC upregulation in the liver (Fig. 3A and B).

IP reduced nNOS and iNOS expression after cerebral ischemia

Cortical and striatal nNOS expression was significantly reduced in the IP group after cerebral ischemia (R23) compared to the EC group (Fig. 4A and B). iNOS expression was also decreased in the cortex, but not in the striatum, by IP (Fig.4C and D). However, immunoreactivity of cortical and striatal iNOS expression was significantly reduced in the IP group after cerebral ischemia (R23) compared to the EC group (Fig. 4C and D).

DISCUSSION

IP is one of the most important endogenous mechanisms involved in neuroprotection against ischemic or reperfusion injury [6, 29-31]. Previous studies have reported two preconditioning paradigms: classical and delayed. Classical preconditioning occurs within minutes after a brief triggering stimulus and lasts for several hours. This phenomenon is related to the direct modulation of energy supplies caused by uncoupling of the mitochondria, pH regulation, and Na^+/Ca^{2+} homeostasis that reduces Ca^{2+} uptake into the mitochondria. While less protective than classical preconditioning, delayed preconditioning begins 12~24 hr after the triggering stimulus and persists for 2~3 days. Delayed preconditioning gives rise to protein kinase C activation of nuclear factor kappa B (NF-κB) and other transcription factors and upregulates protective gene products. For example, induction of heat shock proteins reduces the nuclear binding of proinflammatory transcription factors, increases the antioxidant capacity of cells [32], prevents the activation of intrinsic and extrinsic apoptotic pathways [33], and helps repair organs via a chaperone pathway [34]. Regardless of what pathways are induced, the essential consequences are that both delayed and classical preconditioning decrease energy demand of ischemic tissues [35] and ultimately increase cell survival after the insult [36]. Recently, it has been reported that agmatine exerts neuroprotection against ischemic injury in neuronal cell cultures and experimental stroke in vivo [7, 13]. This neuroprotection is associated with decreased NOS activity and expression, as
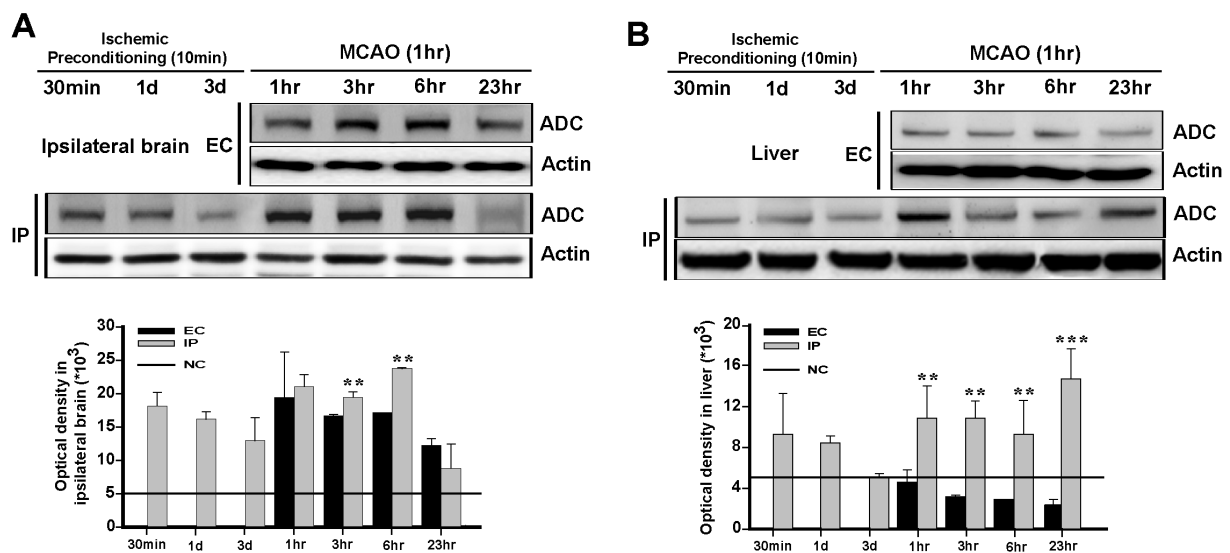

Fig. 3. Immunoblotting of the level of ADC during and after ischemic injury. (A) The expression level of arginine decarboxylase (ADC) in the ipsilateral brain was significantly correlated with the level of agmatine in the brain after IP. (B) Moreover, the expression level of ADC in the liver was significantly increased compared to EC after MCAO. EC, experimental control; IP, ischemic preconditioning; 30 min, 30 min after IP; 1 d, 1 day after IP; 3 d, 3 day after IP; 1 hr, 1 hr after reperfusion; 3 hr, 3 hr after reperfusion; 6 hr, 6 hr after reperfusion; 23 hr, 23 hr after reperfusion (**p<0.05 vs. EC; ***p<0.01 vs. EC).
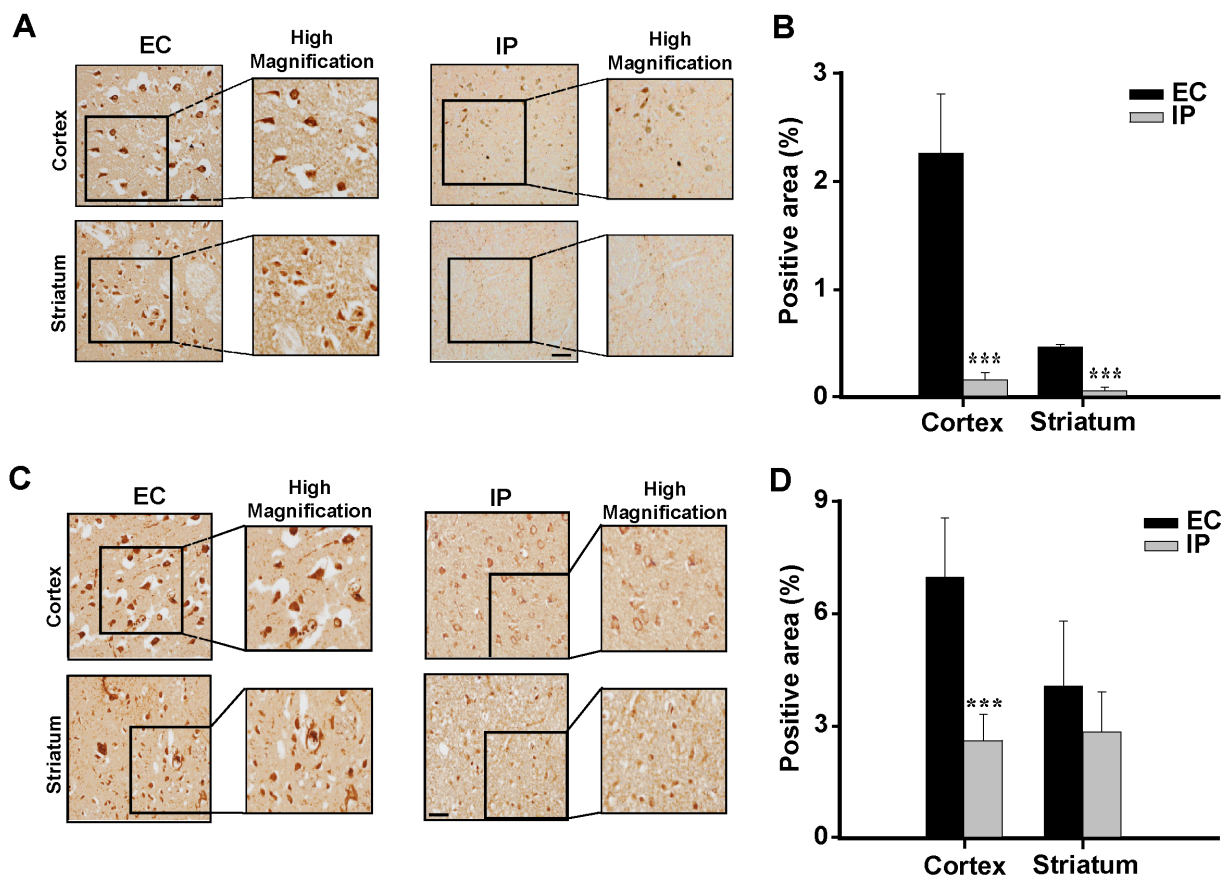
Fig. 4. Immunohistochemistry of nNOS and iNOS in the ischemic rat brain. (A) nNOS-positive cells (brown) in the ipsilateral brain were significantly decreased in the IP group compared with the EC group at 23 hr after reperfusion. (B) Quantification of nNOS-positive cells were analyzed by Image J. The nNOS-positive area in cortex and striatum was significantly decreased in the IP group compared to the EC group at 23 hr after reperfusion. (C) The expression of iNOS-positive cells (brown) in the IP group was significantly decreased in the cortex and striatum at 23 hr after reperfusion. (D) Quantification of nNOS-positive cells in the cortex was significantly different between the EC and IP groups, but not in the striatum at 23 hr after reperfusion. EC, Experimental control; IP, Ischemic preconditioning, Scale bar is 50 μm (***$p < 0.01$ vs. EC).

well as NO generation [7]. In our previous investigations, we demonstrated that exogenous administration of agmatine rescues retinal ganglion cells from hypoxia and tumor necrosis factor-alpha (TNF-α)-induced apoptosis [37, 38]. Agmatine was also reported to be involved in increased intracellular tolerance against stress conditions, and endogenously synthesized agmatine is increased in response to cold-restraint stress [39].

Agmatine is synthesized endogenously by ADC from transported arginine in mitochondria. The ADC activity varies substantially across organs, with the highest activities being detected in the liver, kidney, and stomach and the lowest activities in the brain and adrenal tissues [40]. Moreover, oral administration of agmatine is absorbed from the gastrointestinal tract and readily distributed throughout the body [41], with a limited amount crossing the blood-brain barrier (BBB) [42]. Agmatine analogs that are able to cross the BBB have been developed [43]. Previous reports showed that agmatine is stored in synaptic vesicles, accumulated by ac-

tive uptake, released by depolarization, and catabolized to form putrescine by agmatinase. In the liver and other peripheral tissues, agmatine is alternatively oxidized by diamine oxidase. It is then converted by aldehyde dehydroxylase to form guanido-butanoic acid, which is readily excreted from the body [12].

In this study, we demonstrated that the systemic level of agmatine is increased after IP. Furthermore, we suggest a role of agmatine in ischemic tolerance. A question was raised regarding the changes of the agmatine level in the brain after I/R injury; our data suggest that the level of agmatine in the brain was significantly increased by IP, yet the level of agmatine was lower in the IP group than in the EC group 23 hr after I/R injury. This finding could let us explain the time-dependent changes in the agmatine level, which may be due to an early response in initial I/R injury that was critical to the neuroprotective effect in the IP group. However, the level of agmatine in the EC group was significantly increased 23 hr after I/R injury, which might indicate a delayed protective response

in secondary I/R injury. However, the neurological outcome was significantly reduced in the EC group. Another question that was raised was regarding the ADC expression; our data suggest that the ADC expression during IP and ischemic insult was significantly changed in the ipsilateral brain and liver. The significant changes occurred alongside with the increased agmatine level in the plasma and brain at 30 min after IP. Moreover, the ratio of L-arginine in the liver was significantly reduced at 30 min after IP. These results suggest that liver ADC actively metabolized L-arginine and secreted agmatine into the plasma after the early phase of IP. Further, these results suggest that the local increase of agmatine level was not only a consequence of the translational increase of ADC expression in the brain but also a result of the systemic induction of ADC expression triggered by IP.

The third question was raised concerning how IP in the brain could have systemic effects, including in the liver. Previous reports suggest that the benefits of IP on lung IR-induced systemic inflammatory response may reflect the protection of both the lungs and remote organs. In remote ischemic preconditioning (RIPC), brief ischemia of one organ confers protection to distant organs without direct stress to the organ. Experimental studies have demonstrated that brief I/R of the limb, gut, mesentery, or kidney reduces myocardial infarct size [44]. Furthermore, clinical research has shown that skeletal IP induces myocardial protection [44]. Two reports have shown that limb IP has a protective role against hepatic IR injury in rats [44, 45].The underlying mechanisms and pathways of RIPC are not well established, and an overlap between the neurogenic and humoral pathways has been proposed [44]. Remote cytokine (including TNF-α [46, 47], interleukin-1 [IL-1] [46], and monocyte chemoattractant protein-1 [MCP-1] [48]), NF-κB [49], and iNOS [49] modulation through these signaling pathways has been suggested as a potential mechanism for RIPC-induced protection. It is tempting to speculate that the benefits of IP on ischemia-induced systemic inflammatory response may reflect the protection of both the target organ and remote organs [50]. These reports suggest that the IP-induced systemic pro-inflammatory response and anti-apoptotic pathways may reflect the protection of both the brain and remote organs. In this study, we proposed that ADC and agmatine—typically expressed in the brain and liver— might be systemically upregulated. Therefore, IP could provide systemic protection by upregulating agmatine in the cerebral and peripheral system for ischemic tolerance after IP.

From these findings, we suggest that IP triggers endogenous agmatine synthesis in the brain and liver, which reduces the brain infarct area and edema formation and attenuates the detrimental effects of ischemic injury by suppressing nNOS and iNOS expression.

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