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A Simple Method for Predicting Hippocampal Neurodegeneration in a Mouse Model of Transient Global Forebrain Ischemia

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In the present study, we developed a simple method to predict the neuronal cell death in the mouse hippocampus and striatum following transient global forebrain ischemia by evaluating both cerebral blood flow and the plasticity of the posterior communicating artery (PcomA). Male C57BL/6 mice were anesthetized with halothane and subjected to bilateral occlusion of the common carotid artery (BCCAO) for 30 min. The regional cerebral blood flow (rCBF) was measured by laser Doppler flowmetry. The plasticity of PcomA was visualized by intravascular perfusion of India ink solution. When animals had the residual cortical microperfusion less than 15% as well as the smaller PcomA whose diameter was less than one third compared with that of basilar artery, neuronal damage in the hippocampal subfields including CA1, CA2, and CA4, and in the striatum was consistently observed. Especially, when mice met these two criteria, marked neuronal damage was observed in CA2 subfield of the hippocampus. In contrast, after transient BCCAO, neuronal damage was consistently produced in the striatum, dependent more on the degree of rCBF reduction than on the plasticity of PcomA. The present study provided simple and highly reproducible criteria to induce the neuronal cell death in the vulnerable mice brain areas including the hippocampus and striatum after transient global forebrain ischemia.

Key Words: Global forebrain ischemia, Posterior communicating artery, Laser Doppler flowmetry, Hippocampus, Mouse, C57BL/6

INTRODUCTION

Transient global ischemia has been one of the most interesting pathological conditions in stroke studies. Transient forebrain ischemia followed by reperfusion induces delayed neuronal cell death in several regions of the brain, including the hippocampal CA1 subregion (Pulsinelli & Brierley, 1979). Glutamate excitotoxicity, oxidative stress, inflammation, and apoptosis have been known to be involved in the mechanisms of delayed neuronal death following forebrain ischemia (Lipton, 1999; Lo et al, 2003; Chan, 2004). Rodent models have been central to the elucidation of those pathophysiological mechanisms of global forebrain ischemia. Among them, a mouse model with bilateral common carotid artery occlusion (BCCAO) has become an accepted model for study of neuroprotective mechanisms of action (Olsson et al, 2003; Carswell et al, 2004; Lee et al, 2004).

C57BL/6 mouse strain has an incomplete circle of Willis with a high frequency of posterior communicating artery (PcomA) atresia (Yang et al, 1997; Kitagawa et al, 1998; Wellons et al, 2000). Therefore, as in the gerbil (Mayevsky & Breuer, 1992), only BCCAO in C57BL/6 mice results in disconnection between anterior and posterior circulations of the brain and leads to global forebrain ischemia. In these species, BCCAO without hypotension is sufficient to generate a dense ischemic insult with resultant neuronal necrosis in selectively vulnerable regions of the forebrain (Sheng et al, 1999). Because the plasticity of the PcomA can be evaluated only after the brains are removed from the experimental animals, though it influences cerebral blood flow during the BCCAO, one more criterion is needed to predict the induction of global forebrain ischemia during the BCCAO. Therefore, we developed a procedure to validate a mouse BCCAO model of transient global forebrain ischemia using both laser-Doppler flowmetry and evaluation of the plasticity of PcomA.

METHODS

Induction of global forebrain ischemia

All animal procedures were approved by the Catholic Ethics Committee of the Catholic University of Korea and were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23). Global forebrain ischemia was induced in male C57BL/6 mice weighing 20~24 g (8~9 weeks old). Mice were anesthetized with 1% halothane in

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ABBREVIATIONS: BCCAO, bilateral occlusion of the common carotid artery; LDF, Laser Doppler flowmetry; PcomA, posterior communicating artery; rCBF, regional cerebral blood flow.

a 70% nitrous oxide and 30% oxygen mixture using a face mask during BCCAO for 30 min. Through midline incision, both common carotid arteries were exposed and were temporarily clamped with micro serrefines. After reperfusion, the animals were cared in a warm incubator (32~33°C) until they were sacrificed three days later. During the surgical procedure, rectal temperature was maintained between 36.5~37.9°C with a heating lamp.

Measurement of regional cerebral blood flow (rCBF

Cortical microperfusion was monitored using laser Doppler flowmetry (LDF; Perimed, PF5010, Järfälla, Sweden) from anesthetic induction through 5 min after reperfusion. A flexible probe (407) was attached on the intact skull, 3.5 mm right from the bregma with cyanoacrylate adhesives. The change of cerebral blood flow was calculated as a percentage of values measured for one minute immediately after BCCAO over the baseline values.

Evaluation of the PcomA plasticity and tissue preparation

On the third day after reperfusion, animals were euthanized by an i.p. injection of 15% chloral hydrate and transcardially perfused with normal saline, followed by 4% paraformaldehyde in 0.1 M phosphate buffered solution (pH 7.4). Prewarmed 5 ml of India ink solution which was a mixture of gelatin, mannitol and India ink were infused into the heart and the animals were ice-cooled for 30 min. The whole brain was taken by a digital camera (EOS 300D, Canon, Tokyo, Japan), followed by post-fixation in 4 % paraformaldehyde solution for 4 h. The degree of plasticity of PcomA was evaluated by comparing the diameter of the PcomA with that of the basilar artery using Image-Pro Plus software (version 5.1; MediaCybernetics, Silver Spring, MD). The brain samples were dehydrated with 30% sucrose in 0.1 M phosphate buffer and then rapidly frozen with liquid nitrogen after embedded in Tissue-Tek (Sakura Finetechnical, Tokyo, Japan). Every 5th sections were cut using a cryotome to assess ischemic neuronal injury.

Assessment of neuronal damage

Neuronal damage was visualized by cresyl violet staining. Slides were hydrated from absolute alcohol to tapped water and incubated with 0.1% cresyl violet solution for 20 min. Excessive staining was removed by immersing the slides briefly into 95% alcohol solution and dehydrated. After clearing with 2 series of xylene solution, the sections were mounted with Canada balsam and observed under the light microscopy (BX51, Olympus, Tokyo, Japan).

Because of variable neuronal damage observed after transient global forebrain ischemia in mice, semi-quantitative evaluations of damaged neurons have been performed in the mouse brain (Murakami et al, 1998; Sasaki et al, 2003). The severity of neuronal damage was evaluated by an examiner blinded to the experimental condition and was semi-quantitatively measured as no ischemic neurons (Grade 0), ischemic neuronal damage less than 30% (Grade 1), 31-64% of ischemic neuronal damage (Grade 2), 65-100% of ischemic damage (Grade 3) in the hippocampal subregions (CA1-CA4) and striatum.

Statistics

Semi-quantitative data of ischemic damage were analyzed with Friedman two-way analysis of variance and a post hoc non-parametric multiple comparison test. A p-value of less than 0.05 was considered as statistically significant.

RESULTS

rCBF measurements were made during the operation and change of rCBF was calculated as a ratio of 1 min trace after BCCAO over the baseline values (Fig. 1). In nineteen mice, the percentage of rCBF during BCCAO varied from 3.7 to 60.6% of the baseline. In thirteen (68%) among them, rCBF decreased below 15% of baseline during BCCAO (Table 1).

The plasticity of PcomA was assessed using transcardiac India ink injection and graded on a scale of 0 (underdeveloped) to 1 (well-formed) (Fig. 2). In this classification, among nineteen mice examined, nine mice (47.4%) with underdeveloped PcomA and reduced rCBF less than 15% of the baseline showed consistent neuronal damage in the hippocampal subfields after transient BCCAO (Table 1). In contrast, six out of nineteen mice (31.6%) had well-formed PcomA, showing rCBF more than 15% of baseline during BCCAO. In those cases, no neuronal damage was observed

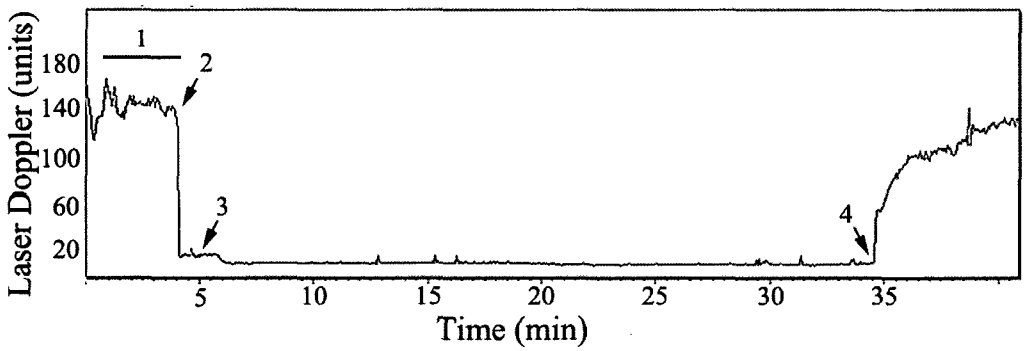

Fig. 1. Representative example of regional cerebral blood flow (rCBF) recording. 1: baseline; 2: bilateral common carotid arteries occlusion (BCCAO) begins; 3: 1 min after BCCAO begins; 4: reperfusion begins. Percentage of rCBF was calculated as a ratio of values traced for 1 min after BCCAO expressed as a period between 2 and 3 over the baseline values.

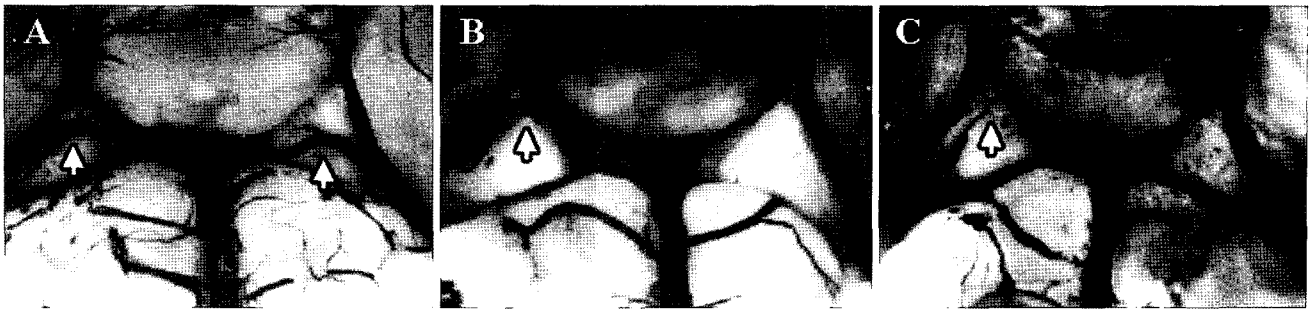

Fig. 2. Brain vasculature of the posterior communicating artery (PcomA) in C57BL/6. (A) The PcomAs with the diameter of more than one third of that of basilar artery are identified on both sides. (B) The PcomA is absent on the left side whereas a large PcomA is patent on right side. (C) A narrow anastomosis is shown between the PcomA and the basilar artery on the right side whereas anastomosis is absent on the left side.

Table 1. Relationship among cerebral blood flow for 1 min after ischemia, plasticity of posterior communicating artery, and ischemic neuronal damage

Case	Parameters		Ischemic neuronal damage					
	PcomA	rCBF	mCA1	lCA1	CA2	CA3	CA4	CP
1	+	60.6%	X	X	X	X	X	X
2	+	25.9%	X	X	X	X	X	O
3	+	25.0%	X	X	X	X	O	O
4	+	24.7%	X	X	O	O	O	O
5	+	23.2%	X	X	O	O	O	O
6	+	23.0%	X	X	X	X	O	O
7	+	13.5%	X	X	X	X	O	O
8	+	9.3%	X	X	X	X	O	O
9	+	8.8%	X	X	O	O	O	O
10	+	5.7%	X	X	O	O	O	O
11	−	14.5%	O	O	O	O	O	O
12	−	12.2%	O	O	O	O	O	O
13	−	10.3%	O	O	O	O	O	O
14	−	9.4%	O	O	O	O	O	O
15	−	9.2%	O	O	O	O	O	O
16	−	8.3%	O	O	O	O	O	O
17	−	7.9%	X	X	O	O	O	O
18	−	6.9%	O	O	O	O	O	O
19	−	3.7%	O	O	O	O	O	O

rCBF: cerebral blood flow, PcomA: posterior communicating artery, mCA1: medial CA1 of the hippocampus, lCA1: lateral CA1 of the hippocampus, CP: caudoputamen. PcomA plasticity; −: PcomA with no anastomosis or less than one third of he diameter of the basilar Artery, +: PcomA with more than one third of the diameter of the basilar artery. Ischemic neuronal damage; X: no damaged neurons, O: damaged neurons more than grade 1 (see Methods).

in all hippocampal subfields at three days after transient BCCAO. Interestingly, among thirteen mice showing rCBF less than 15% of the baseline during BCCAO, four mice (30.8%) had well-formed PcomA: in those four animals, transient BCCAO resulted in no neuronal damage in all hippocampal subfields. In all mice subjected to transient BCCAO except one showing less rCBF reduction, neuronal damage was observed in the striatum and being worse with the tendency of PcomA atresia.

Histological damage was evident in the pyramidal cell layers of the hippocampus and the striatum at 3 days after 30 min of BCCAO (Fig. 3). In mice with underdeveloped PcomA, neuronal cell death was apparent in CA1, CA2, and CA4 of the hippocampus, and the striatum, but not in the CA3 of the hippocampus, while in mice with well-formed PcomAs, intact neurons in all subfields of the hippocampus and the striatum remained.

Neuronal damage in the hippocampus and the striatum was expressed semi-quantitatively on a scattered plot (Fig. 4). The proportion of grade 0 neuronal damage was more decreased as the stricter criteria was applied. In other words, mice with both underdeveloped PcomA and rCBF less than 15% of the baseline had more severe ischemic damage than those with the rCBF criteria only. Moreover, Friedman test showed that ischemic neuronal damage in CA2 subfield of the hippocampus was significantly severe than lateral CA1 or CA3 subregion.

DISCUSSION

Models of transient global cerebral ischemia in gerbil and rat have been extensively used in order to investigate the pathophysiology of ischemic injury and identify new drugs for the treatment of stroke. While manipulating gene expression in these species is complicated, genetically engineered mice are being successfully utilized to evaluate the role of single genes and their gene products in the pathophysiology of cerebral ischemia (Martinou et al, 1994; Panahian et al, 1996; Kondo et al, 1997). Although the mouse models of transient global forebrain ischemia have been used by several lines of investigators (Fujii et al, 1997; Wellsons et al, 2000; Yang et al, 2000), this model has not fully established in terms of reproducibility of ischemic injury in the vulnerable brain areas. In the present study, we developed a simple procedure to predict the hippocampal and striatal neuronal degeneration in a mouse BCCAO model of transient global forebrain ischemia by evaluating rCBF during BCCAO and the plasticity of PcomA at the end of animal experiments.

Because laser Doppler flowmetry works by illumination of selected tissue, there may be substantial variability in measurements of rCBF by this methodology in neighboring regions due to temporal variations in blood flow as well as

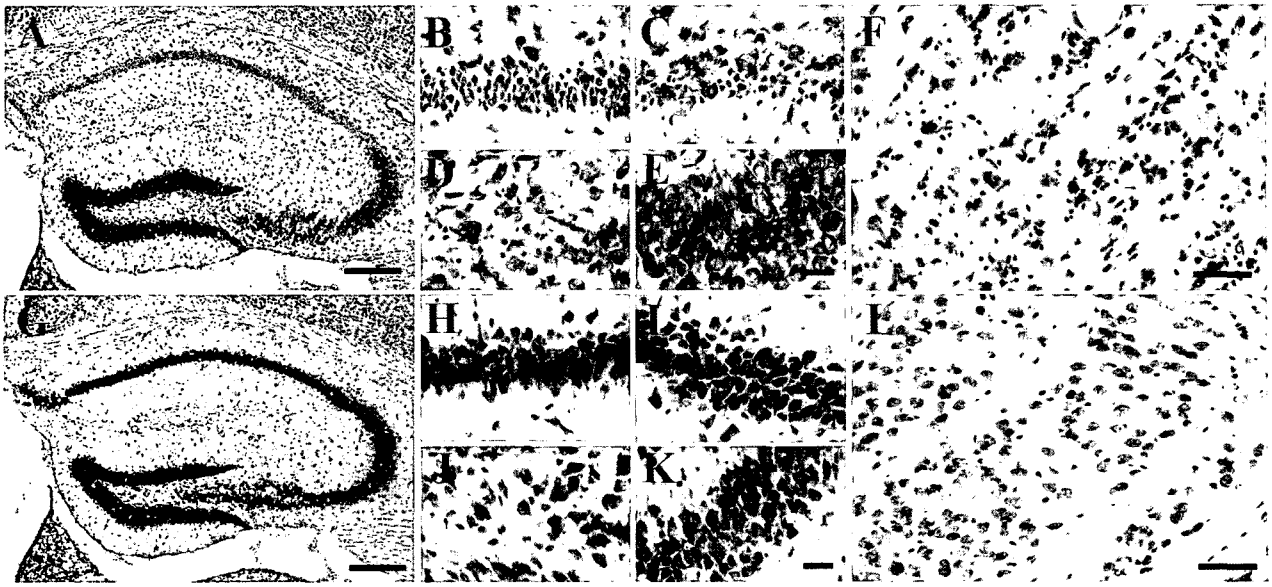

Fig. 3. Cresyl violet staining of the hippocampus and striatum after 30 minutes of BCCAO and reperfusion for 3 days. (A-F) Neuronal cell death in the hippocampus and the striatum from the mouse with underdeveloped PcomA. The CA1 (B), CA2 (C), CA4 (D) of the hippocampus and the striatum (F) show ischemic neuronal change, but CA3 (E) of the hippocampus shows little change. (G-L) Neuronal cell death in the hippocampus and the striatum from the mouse with large PcomAs. The CA1 (H), CA2 (I), CA3 (K), CA4 (J) of the hippocampus and striatum (L) show a lot of intact neurons. Scale bar in A and G = 500 μm. Scale bar in E and K = 50 μm. Scale bar in F and L = 50 μm.

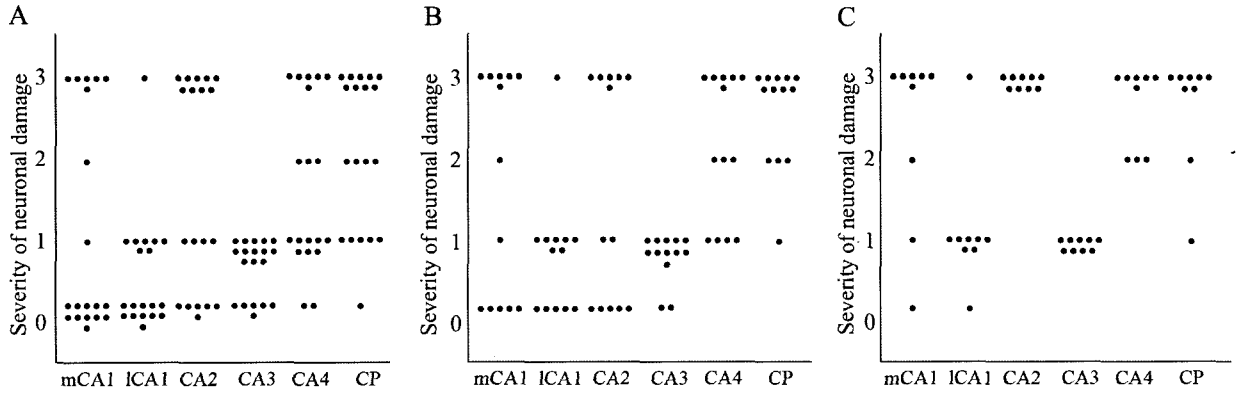

Fig 4. Scattered plot of severity of neuronal damage in the hippocampus and the striatum. Note that proportion of grade 0 neuronal damage is being decreased as mice with rCBF less than 15% of the baseline are selected (B) and both criteria of rCBF and PcomA plasticity are applied (C). Ischemic neuronal damage is graded as follows; 0: no damaged neurons; 1: damaged neurons less than 30%; 2: 31~64% damaged neurons; 3: 65~100% damaged neurons. mCA1: medial CA1 of the hippocampus, lCA1: lateral CA1 of the hippocampus, CP: caudoputamen. Significant differences were found in a non-parametic multiple comparison test (CA2 vs. lCA1, $p<0.05$; CA2 vs. CA3, $p<0.05$).

spatial heterogeneity in the tissues (Kempski et al, 1995). Thus, we measured rCBF transcranially over the right hemisphere (3.5 mm lateral from midline at the level of bregma) during the operation. We found that if rCBF during 1 min after BCCAO was less than 15% of preischemic baseline, all mice showed ischemic neuronal injury in at least the striatum. Therefore, this degree of reduction in rCBF (less than 15% of the baseline) during BCCAO seems to be suitable as an inclusion criterion in selecting experimental mice for the neuroprotection studies. But this criterion' s not sufficient enough to set up a mouse BCCAO model because transient BCCAO resulted in no neuronal damage in at least CA1 hippocampal subfield in mice which had well-formed PcomA, in spite of their rCBF showing less than 15% of the baseline.

In the present study, the mouse model of transient global forebrain ischemia has been developed in the C57BL/6 strain which is the most common background strain of genetically modified mice (Tsuchiya et al, 2002). It has been demonstrated that C57BL/6 mice were significantly more susceptible to global ischemic injury and that the vulnerability could be accounted for the presence of a circle of Willis

lacking vascular connections between the anterior and posterior circulations during BCCAO (Fujii et al, 1997; Yang et al, 1997). However, McColl et al (2004) reported that, among ten C57BL/6 mice investigated, only three mice were deficient for both PcomAs and six mice were absent for one PcomA. In addition, Wellons et al (2000) demonstrated that SV129 mice were remarkably resistant to transient forebrain ischemia as compared to C57BL/6 strain, which was independent of the magnitude of CBF reduction. This study also showed that C57BL/6 mice had profoundly smaller PcomAs than SV129 mice. Considered together, these results suggest that the hypoplasticity of PcomA is a reliable indicator of ischemic injury to transient BCCAO in the C57BL/6 strain mice. Therefore, we raise the concept that mice with well-formed PcomA, as an exclusion criterion, should be ruled out in the studies of neuroprotection using this model.

In rat and gerbil, the pyramidal neurons in CA1 subfield including the lateral segment were reportedly the most vulnerable to transient global forebrain ischemia (Kirino, 1982; Pulsinelli et al, 1982). In contrast, our results showed that CA2 subfield was the most vulnerable region among the pyramidal sectors of the hippocampus after transient BCCAO in mice. This finding is accordance with a previous report by Yang et al (2000) showing that the lateral segment of the CA1 subfield in the murine hippocampus was relatively resistant to ischemic injury.

In the present study, striatal neuronal injury was consistently produced in eighteen out of nineteen mice examined at 3 days after transient BCCAO. Contrast to the hippocampal findings, degree of ischemic neuronal damage was more related with rCBF reduction than plasticity of PcomA. Related to our results, Terashima et al (1998) reported that striatal injury after transient global ischemia was reproducible in C57BL/6 mice. Taken together with our hippocampal findings, these results suggested that hippocampal injury was likely to be more sensitive to the PcomA plasticity than striatal injury to ischemic insult.

In conclusion, we developed a procedure to validate a mouse BCCAO model of transient global forebrain ischemia. These results support the belief that use of C57BL/6 mice in studies of neuroprotection requires both rCBF measurement during BCCAO and evaluation of the plasticity of PcomA at the end of animal experiments as an inclusion criterion and an exclusion criterion, respectively.

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