

The Effects of Glutamate NMDA Receptor Antagonist MK-801 on Gastrointestinal Motility after Middle Cerebral Artery Occlusion in Rats

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This study was performed to investigate the role of glutamate neurotransmitter system on gastrointestinal motility in a middle cerebral artery occlusion (MCAO) model of rats. The right middle cerebral artery was occluded by surgical operation, and intestinal transit and geometric center as a parameter of gastrointestinal motility and expression of c-Fos protein in the insular cortex and cingulate cortex were measured at 2 and 12 h after MCAO. Intestinal transit was $66.3 \pm 7.5\%$ and $62.3 \pm 5.7\%$ 2 and 12 h after sham operation, respectively, and MCAO significantly decreased intestinal transit to $39.0 \pm 3.5\%$ and $47.0 \pm 5.1\%$ at 2 and 12 h after the occlusion, respectively ($p < 0.01$). The geometric center was 5.6 ± 0.4 and 5.2 ± 0.9 at 2 and 12 h after sham operation, respectively, and MCAO significantly decreased geometric center to 2.9 ± 0.8 and 3.0 ± 0.3 at 2 and 12 h after the occlusion, respectively ($p < 0.01$). In control animals, injection of atropine decreased intestinal transit to $35.9 \pm 5.2\%$, and injection of glutamate NMDA receptor antagonist, MK-801, decreased intestinal transit to $28.8 \pm 9.5\%$. Pretreatment with MK-801, a glutamate NMDA receptor antagonist, in the MCAO group decreased intestinal transit to $11.8 \pm 3.2\%$, which was significantly decreased compared to MCAO group ($p < 0.01$). MCAO markedly increased the expression of c-Fos protein in the insular cortex and cingulate cortex ipsilateral to the occlusion 2 h after MCAO, and pretreatment with MK-801 produced marked reduction of c-Fos protein expression compared to MCAO group ($p < 0.01$). These results suggest that modulation of gastrointestinal motility after MCAO might be partially mediated through a glutamate NMDA receptor system.

Key Words: Glutamate, Insular cortex, Intestinal transit, MCAO, MK-801

INTRODUCTION

Patients suffering from acute stroke are at risk of developing a wide range of complications secondary to stroke. Cerebrovascular accidents cause autonomic dysfunction including cardiovascular and gastrointestinal disorders as well as sensori-motor disorders. Many basic studies on autonomic function after a stroke have been performed to examine cardiovascular disorders [1,2]. Cerebrogenic cardiovascular disturbances and sudden death are induced by the insula after stroke [1]. And stroke patients showed a parasympathetic cardiac deficit and increased sympathetic cardiovascular modulation [3]. However, very little attention has been paid to gastrointestinal motility in autonomic function after stroke. Constipation is recognized as a serious problem in clinical practice, affecting 60% of stroke rehabilitation ward attendees [4]. Although Bracci et al. [5]

reported that constipation is due to neurological lesions and is unrelated to age, gender and physical inactivity, the mechanism of constipation after stroke has not thus far been well-studied.

Gastrointestinal function is under the control of the autonomic nervous system through intrinsic and extrinsic autonomic inputs that modulate intrinsic autonomic functions. Contractions of the longitudinal muscle evoked by electrical stimulation of the myenteric nerves or by glutamate have been shown to be significantly reduced by an NMDA receptor antagonist [6]. The dorsal motor nucleus of the vagus (DMV) supplies parasympathetic motor preganglionic fibers to the viscera. A group of neurons located in the insular cortex projects directly to the specific gastric portion of the dorsal vagal complex in rats [7]. Electrical stimulation to the insular cortex produces changes in autonomic functions and gastrointestinal motility [8]. These studies indicate that the insular cortex modulates gastrointestinal function through indirect neural circuits. Also the cingulate cortex provides output to autonomic output structures and to the amygdala [9].

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ABBREVIATIONS: MCAO, middle cerebral artery occlusion; NMDA, N-methyl-D-aspartic acid; DMV, dorsal motor nucleus of the vagus; GI, gastrointestinal.

The insular cortex and cingulate cortex receive blood supply from the middle cerebral artery, which originates at the division of the internal carotid artery. The occlusion of the middle cerebral artery (MCAO) is most frequently used for induction of stroke in animal models [10]. Animal models of focal cerebral ischemia allow study of the pathophysiology, evaluation of complications, and therapeutic approaches in stroke. In order to evaluate gastrointestinal motility in humans, electrogastrography using cutaneous electrodes [11] and radiological study [12] were used. However, measurement of gastrointestinal transit is a widely used technique for evaluating gastrointestinal motility in animals and is quantified by measuring the movement of radioactive markers or dyes that have been instilled into the stomach [13].

Glutamate is the major excitatory neurotransmitter in the central nervous system and is also an excitatory neurotransmitter in the enteric nervous system [6]. Since high levels of glutamate in plasma and cerebrospinal fluid have been demonstrated in patients with acute ischemic stroke [14], it is postulated that glutamate neurotransmitter system could be involved in gastrointestinal motility after MCAO.

In this study, the role of glutamate neurotransmitter system on gastrointestinal motility was investigated in a MCAO model of rats. Gastrointestinal transit as a parameter of gastrointestinal motility and c-Fos protein in the insular cortex and cingulate cortex as a neural marker were measured after MCAO. In addition, the effect of MK-801, a glutamate NMDA receptor antagonist, on gastrointestinal transit and c-Fos protein expression was investigated to elucidate treatment of constipation after stroke.

METHODS

Experimental animals

Fifty-eight adult male Sprague-Dawley rats (Saemtaco Biokorea Co, Suwon, Korea) weighing 250 g were used. Animals were group-housed in a thermoregulated environment with a 12 h light/dark cycle. They were fasted for 48 h with free access to water only until use. Experimental animals were divided into control and MCAO groups. The control group was subdivided into control group for atropine injection or MK-801 injection and sham group for MCAO. MCAO group was subdivided into MCAO group and pretreatment with MK-801 group. The Institutional Ethical Committee on the Experimental Use of Animals approved all procedures.

MCAO

Animals were anesthetized with 5% isoflurane (Ilsung Pharmaceutical Co, Seoul, Korea) delivered in air for 2 min as an induction, and anesthesia was maintained with 2~3% isoflurane delivered in air at 0.5 l/min during surgery. Body temperature was continuously monitored with a rectal probe and maintained at $37.0 \pm 0.5^\circ\text{C}$ during surgery and 2 h after MCAO. The model for permanent focal brain ischemia was produced by occlusion of the right MCA according to the method of Laing et al. [15] with minor modifications. Briefly, the right common carotid artery (CCA), internal carotid artery (ICA) and external carotid artery (ECA) were exposed through a midline incision of the neck. The ECA

was isolated, and the superior thyroid and occipital arteries were cut off with electric cautery. The distal portion of the ECA was ligated with 5-0 sutures. The CCA, ICA and pterygopalatine branch of the ICA were temporarily clipped with microclamps and then, through an incision, an arteriotomy was performed near the distal portion of the ECA. Through the incision at the ECA, a 4-0 silicone-coated nylon suture with thermally treated tip was introduced. The suture tip was advanced into the ICA 16~18 mm beyond the carotid bifurcation until mild resistance was met, indicating the tip was lodged in the anterior cerebral artery and thus blocking blood flow to the MCA. The ECA was ligated with a 5-0 suture near the carotid bifurcation. Finally, microclamps on the CCA and pterygopalatine artery were released and the incision of the skin was sutured. In the sham group, the CCA, ICA and ECA of the right side were exposed through a midline incision of the neck and the incision of the skin was sutured.

Drug injection

Animals received a single intraperitoneal injection of MK-801 (Research Biomedicals Inc, Natick, MA, USA) dissolved in sterile saline (1.0 mg/ml) with dosage of 1.0 mg/kg 30 min before MCAO. Atropine sulfate (Sigma, St. Louis, MO, USA) was injected intraperitoneally in a dose of 0.1 mg/kg. Intestinal transit was measured 60 min after atropine or MK-801 injection in control animals.

Measurement of intestinal motility

Physiological saline containing Evans blue 0.15 ml (50 ml/kg) was orally ingested into the stomach 2 and 12 h after surgery via an animal feeding needle with a straight blunt end (G18, Popper's & Sons Inc, New Hyde Park, NY, USA). Additional air (0.5 ml) was added to flush the residual dye remaining in the feeding needle into the rat. After 30 min of dye ingestion, the animals were perfused under deep anesthesia with 1% paraformaldehyde solution to fix the gastrointestinal system; animals were placed under deep anesthesia with an intraperitoneal injection of pentobarbital sodium (100 mg/kg). The stomach and small intestine were exposed immediately by a laparotomy. After ligation of gastroduodenal and ileocecal junctions and the separation of the ligaments and mesentery, the whole stomach and small intestine were carefully mobilized from the abdominal cavity and placed on a wet wooden board to observe the leading edge of Evans blue in the intestine. The small intestinal length and the length from the gastroduodenal junction to the Evans blue front were measured with a ruler. Intestinal transit was represented as the percentage of the length of small intestine in which Evans blue moved divided by the total length of the small intestine.

To measure the geometric center, the small intestine was divided into 10 equal segments. Each segment was crushed and dissolved in 15 ml of 0.1 N NaOH for 30 sec, then another 15 ml of 0.1 N NaOH was added and homogenized for 30 sec. The suspension was allowed to settle for 1 h at room temperature, and 1 ml of the supernatant was centrifuged for 20 min at 1,300 rpm. Absorbance of Evans blue in 0.1 ml of the centrifuged solution from each segment of the small intestine was measured using a spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). The sample absorbance was read at a wavelength of 565 nm and expressed as the optical density. A standard dilution curve

was obtained in each experiment relating the Evans blue concentration to the optical density of 0.1 N NaOH. The geometric center was calculated by summation of the percentage of absorbance measured in each segment multiplied by the segment number, that is, geometric center = \sum (fraction of Evans blue per segment \times segment number) [13,16].

Measurement of c-Fos protein

Immunohistochemical analysis of c-Fos protein expression was performed as described previously [17]. Under deep anesthesia with an overdose of pentobarbital sodium, the animals were perfused transcatheterially with 250 ml of 0.1 M phosphate buffer (PB), and then perfused with 500 ml of 4% paraformaldehyde dissolved in 0.1 M PB (pH 7.4). The brains were dissected and post-fixed with the same fixative solution for 4 h at room temperature. The fixed brains were immersed in 30% sucrose in phosphate buffered saline (PBS) for 2 days at 4°C for cryoprotection. The tissue was sectioned to a thickness of 40 μ m on a freezing microtome (Leica, Wetzlar, Germany), incubated for 30 min with 6% H₂O₂, rinsed twice for 10 min with 0.1 M PBS that contained 5% dry milk and then incubated with 0.8% Triton X-100 that was dissolved in 0.1 M PBS plus 0.5% bovine serum albumin (BSA) (PBS+BSA). After a brief wash, the tissue was incubated overnight at room temperature with a rabbit polyclonal anti-c-Fos antibody (1 : 1,000) (Oncogene Research Products, San Diego, CA, USA) in PBS+BSA. The following day, the tissue was rinsed with PBS+BSA, incubated with a biotinylated goat anti-rabbit secondary antibody (DAKO, Glostrup, Denmark) and the Elite ABC Kit (Vector Laboratories, Burlingame, CA, USA). Neurons with c-Fos protein nuclei were visualized by incubating the tissue with 0.05% diaminobenzidine HCl (DAB) and 0.003% H₂O₂. After the DAB reaction was completed, the tissue was rinsed with 0.1 M PB, mounted on gel-coated slides, air-dried, dehydrated, xylene-cleared, and coverslipped with Permount (Fisher Chemical, Fair Lawn, NJ, USA). For quantification, c-Fos-positive neurons were counted using a digital image analysis system (Image-Pro; Media Cybernetics Inc, Bethesda, MD, USA) in the cortex.

Statistical analysis

All data are presented as the mean \pm SD. The statistical significance of the differences was assessed using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc comparison matrix. Values of $p < 0.05$ were considered significant.

RESULTS

Effect of MCAO on intestinal motility

Intestinal transit, the distance traveled by the leading edge of Evans blue, was $66.3 \pm 7.5\%$ and $62.3 \pm 5.7\%$, 2 and 12 h after the sham operation, respectively. The intestinal transit in the MCAO group was $39.0 \pm 3.5\%$ and $47.0 \pm 5.1\%$ at 2 and 12 h after MCAO, respectively, which was significantly decreased compared to the sham group ($p < 0.01$). The geometric center, the mean travel distance of dye in 10 segments of the small intestine, was 5.6 ± 0.4 and 5.2 ± 0.9 at 2 and 12 h after MCAO, respectively, in the sham group. The geometric center in the MCAO group was 2.9 ± 0.8 and

3.0 ± 0.3 at 2 and 12 h after MCAO, respectively, which was significantly decreased compared to the sham group ($p < 0.01$). Intestinal transit and geometric center of intestinal motility showed a similar response after MCAO (Fig. 1). This study also shows that data from the geometric center which represents the mean travel distance of dye in 10 segments of the small intestine reinforce the data from the intestinal transit which represents the distance traveled by the leading edge of Evans blue in the small intestine.

Effect of atropine and MK-801 on intestinal motility

To evaluate the effect of cholinergic system and glutamate system on gastrointestinal motility atropine sulfate, MK-801 and saline were injected in control animals. Intestinal transit 2 h after injection of atropine sulfate in control animals was $35.9 \pm 5.2\%$ and after saline injection was $66.3 \pm 5.0\%$. The reduction of intestinal transit in the atropine group was almost equal to the value of the MCAO group but was significantly decreased compared to the saline group ($p < 0.01$). After injection of MK-801 in control animals, the intestinal transit was $28.8 \pm 9.5\%$, which was significantly decreased compared to the saline group ($p < 0.01$) and MCAO groups ($p < 0.05$). Pretreatment with MK-801 in the MCAO group significantly decreased the intestinal transit to $11.8 \pm 3.2\%$ compared to the saline group, atropine group, and MK-801 group ($p < 0.01$) (Fig. 2).

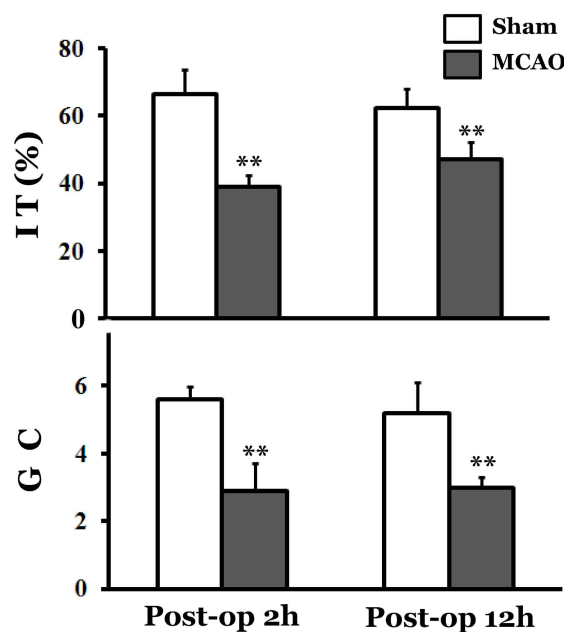


Fig. 1. Changes in intestinal motility 2 and 12 h after MCAO. Sham, the common carotid artery, internal carotid artery and external carotid artery in the right side were exposed through a midline incision of the neck and the incision of the skin was sutured; MCAO, middle cerebral artery occlusion; IT, intestinal transit; GC, geometric center; Post-op, time after MCAO. The number of animals was 7 in each session of each group. Values are mean \pm SD. **Significant differences between Sham and MCAO (** $p < 0.01$).

Effect of MCAO on expression of c-Fos protein in the insular cortex and cingulate cortex

The insular cortex in the sham group did not show any expression of c-Fos protein. In the MCAO group, expression of c-Fos protein was markedly increased in the ipsilateral insular cortex, especially in layers II, III and VI ipsilateral to the occlusion 2 h after MCAO, but the insular cortex contralateral to the occlusion did not show any expression of c-Fos protein. The number of c-Fos positive neurons was $1,057 \pm 153$ in the insular cortex ipsilateral to the occlusion. c-Fos protein was markedly expressed in the ipsilateral cin-

gulate cortex, piriform cortex and motor cortex as well as insular cortex 2 h after MCAO, but was not expressed in the contralateral cortex. The cingulate cortex ipsilateral to the occlusion showed 361 ± 52 of c-Fos positive neurons 2 h after MCAO. However, expression of c-Fos protein could not be measured in the cerebral cortex 12 h after MCAO, since the tissue in the cortex was necrotized at that time. Pretreatment with MK-801 produced marked reduction of c-Fos protein expression in the cerebral cortex 2 h after MCAO. The number of c-Fos positive neurons was 50 ± 20 in the insular cortex after pretreatment with MK-801, which showed the most reduction of c-Fos protein expression in the cerebral cortex. The number of c-Fos positive neurons in the cingulate cortex ipsilateral to the occlusion was 144 ± 33 in the pretreatment with MK-801 group, which was significantly decreased compared to MCAO group ($p < 0.01$) (Fig. 3, 4).

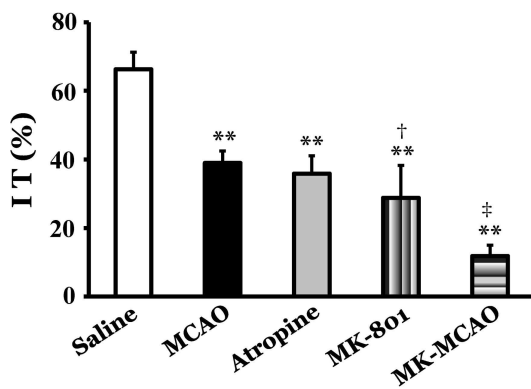


Fig. 2. Effect of MK-801 on intestinal motility after MCAO. Intestinal transit was measured 2 h after injection or surgery. Saline, intraperitoneal injection of sterile saline solution (1.0 ml/kg) in control animals; MCAO, middle cerebral artery occlusion; Atropine, intraperitoneal injection of atropine sulfate in control animals; MK-801, intraperitoneal injection of MK-801 in control animals; MK-MCAO, pretreatment with MK-801 in MCAO. The number of animals was 5 in each group, except MCAO group (n=7, data from Fig. 1). Values are mean±SD. **Significant differences from Saline (** $p < 0.01$). †Significant differences from MCAO († $p < 0.05$, ‡ $p < 0.01$).

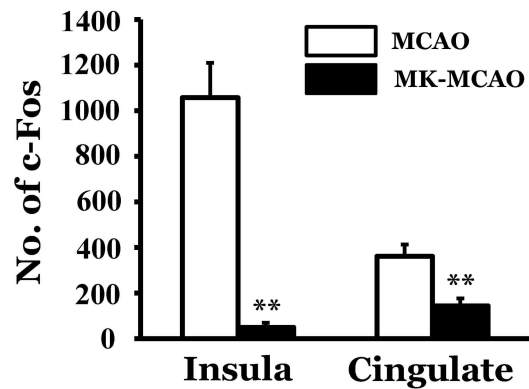


Fig. 3. Effect of MK-801 on expression of c-Fos protein in the insular cortex and cingulate cortex ipsilateral to the occlusion 2 h after MCAO. MCAO, middle cerebral artery occlusion; MK-MCAO, pretreatment with MK-801 in MCAO. The number of animals was 5 in each group. Values are mean±SD. **Significant differences between MCAO and MK-MCAO (** $p < 0.01$).

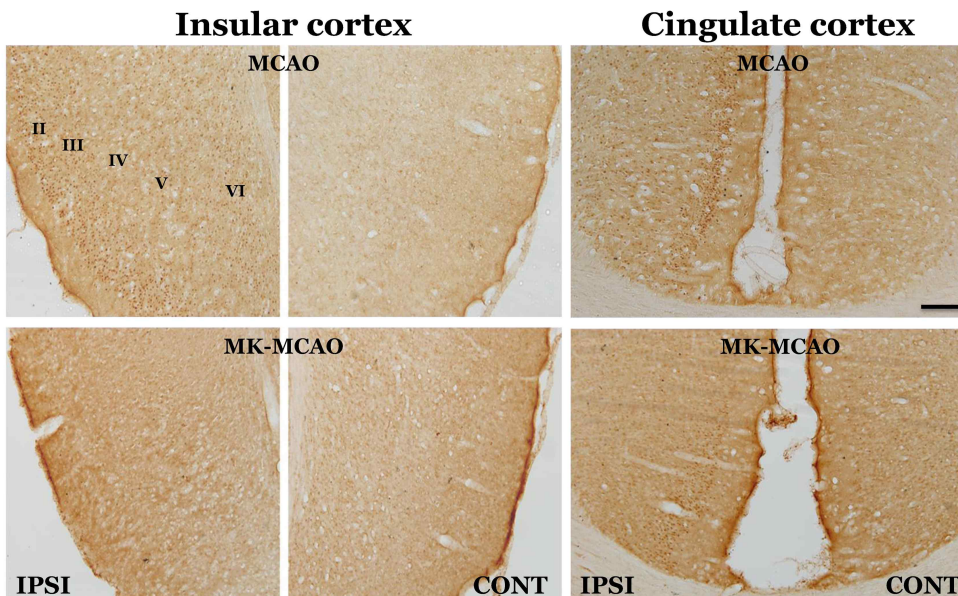


Fig. 4. Photomicrographs of c-Fos protein expression in the bilateral insular cortex and cingulate cortex 2 h after MCAO. MCAO, middle cerebral artery occlusion; MK-MCAO, pretreatment with MK-801 in MCAO; IPSI, ipsilateral to the occlusion; CONT, contralateral to the occlusion. Scale bar: 200 μ m.

DISCUSSION

Ischemia or infarction of the cerebral cortex produces changes in autonomic functions from simple complications to sudden death. Many researchers have focused on the effects of focal cerebral ischemia or infarction on cardiovascular functions and have reported that strokes in the insular cortex were associated with impairment of cardiac autonomic functions [1,3,18]. MCAO causes ischemia and infarction of the territory supplied by its branches, which includes almost all of the cerebral cortex including the insula, basal ganglia and internal capsule. MCAO of the right side was chosen in this study because many studies have suggested that all stroke-related autonomic abnormalities appear more relevant in patients with right-sided hemispheric infarctions [1,18]. The insular cortex is divided into three cytoarchitectonic areas according to functions: a rostroventral agranular insula relating to olfactory and autonomic functions, an intermediate dysgranular insula relating to gustatory functions, and a caudodorsal granular insula relating to somatosensory, auditory and visual functions [19,20]. Stimulation of the anterior insular cortex has been shown to produce gastric motor responses in animal studies [7,21]. These reports indicate that the insular cortex plays a major role in control of gastrointestinal motility as well as cardiovascular function.

In this study, both the intestinal transit and the geometric center in the MCAO group were significantly decreased compared to the sham group. Those results strongly suggest that gastrointestinal motility decreases after focal cerebral ischemia, explaining the high incidence of constipation after strokes. The intestinal transit and the geometric center did not show any significant difference between 2 h and 12 h after MCAO. It is postulated that MCAO causes a permanent impairment of brain function after 2 h of MCAO. Considering that well-established sympathetic hyperfunction in the acute phase of stroke [22], reduction of gastrointestinal motility after MCAO in this study is mainly caused by activation of the sympathetic nervous system. Atropine injection significantly decreased gastrointestinal motility through its antimuscarinic effect. The decreased rate of intestinal transit after atropine injection was similar to the rate of the MCAO group, which suggests that MCAO has a potent effect on the suppression of gastrointestinal motility. Intraperitoneal injection of MK-801 in control animals produced more of a reduction in intestinal transit than MCAO group. Considering that contraction of the longitudinal muscle evoked by glutamate was significantly reduced by an NMDA receptor antagonist [6], the reduction of gastrointestinal motility induced by MK-801 in this study suggests that glutamate acts on the enteric nervous system as well as the cerebral cortex in the control of gastrointestinal motility. Pretreatment with MK-801 in MCAO group produced more of a reduction in intestinal transit than MCAO group, which represents that glutamate facilitates contraction of intestinal muscles after MCAO. Therefore, increased level of glutamate after MCAO may modulate gastrointestinal motility which is decreased by sympathetic hyperfunction.

The insular cortex is connected by direct descending pathways with some bulbar structures, particularly with the DMV that receives gastric afferent inputs from the vagus nerve and contains the gastric preganglionic neurons. Also the DMV participates directly in the gastrointestinal reflex. One study showed that electrical stimulation of a certain

area in the ventral insular cortex had no significant effect on heart and respiration rates but evoked changes in gastric motility [8]. This report supports the idea that the cerebral cortex takes part in modulation of gastrointestinal motility mediated through glutamate NMDA receptor system.

MCAO induced high expression of c-Fos protein in the piriform cortex, cingulate cortex and motor cortex as well as insular cortex ipsilateral to the occlusion, but did not induce expression in the cortex contralateral to the occlusion. This result concurred with extensive induction of immediate early genes in the cerebral cortex caused by focal ischemia [23]. Significantly increased expression of c-Fos protein occurred 2 h after MCAO in the insular cortex and cingulate cortex may be related to stroke-induced gastrointestinal disturbances, since the insular cortex modulates various autonomic functions [8] and the cingulate cortex provides output to autonomic output structures and the amygdala [9]. Ischemia in the brain causes excitotoxic changes in brain tissue [24], and expression of immediate early genes induced by ischemic injury have been shown to be involved in programmed cell death cascades [25]. Excitotoxic damage to the insular cortex caused by MCAO may produce expression of c-Fos protein. Increased expression of c-Fos protein in the insular and cingulate cortex after MCAO does not represent reduction of gastrointestinal motility directly in this study. Pretreatment with MK-801 markedly inhibited expression of c-Fos protein in the cerebral cortex after MCAO, which is corresponding to reduction of the gastrointestinal motility by pretreatment with MK-801. Therefore, these results indicate that the expression was mediated through an NMDA glutamate receptor at acute stage of brain ischemia [23].

Taken together, the findings of this study suggest that modulation of gastrointestinal motility after MCAO might be partially mediated through a glutamate NMDA receptor system. These results may provide a basic strategy for treatment of constipation in stroke patients.

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