Electrical Stimulation of the Cerebral Cortex Exerts Antiapoptotic, Angiogenic, and Anti-Inflammatory Effects in Ischemic Stroke Rats Through Phosphoinositide 3-Kinase/Akt Signaling Pathway

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Background and Purpose—Neuroprotective effects of electric stimulation have been recently shown in ischemic stroke, but the underlying mechanisms remain poorly understood.

Methods—Adult Wistar rats weighing 200 to 250 g received occlusion of the right middle cerebral artery for 90 minutes. At 1 hour after reperfusion, electrodes were implanted to rats on the right frontal epidural space. Electric stimulation, at preset current (0 to 200 μA) and frequency (0 to 50 Hz), was performed for 1 week. Stroke animals were subjected to behavioral tests at 3 days and 1 week postmiddle cerebral artery and then immediately euthanized for protein and immunohistochemical assays. After demonstration of behavioral and histological benefits, subsequent experiments pursued the mechanistic hypothesis that electric stimulation exerted antiapoptotic effects through the phosphoinositide 3-kinase-dependent pathway; thus, cortical stimulation was performed in the presence or absence of specific inhibitors of phosphoinositide 3-kinase (LY294002) in stroke rats.

Results—Cortical stimulation abrogated the ischemia-associated increase in apoptotic cells in the injured cortex by activating antiapoptotic cascades, which was reversed by the phosphoinositide 3-kinase inhibitor LY294002 as reflected behaviorally and immunohistochemically. Furthermore, brain levels of neurotrophic factors (glial cell line-derived neurotrophic factor, brain-derived neurotrophic factor, vascular endothelial growth factor) were upregulated, which coincided with enhanced angiogenesis and suppressed proliferation of inflammatory cells in the ischemic cortex.

Conclusions—These results suggest that electric stimulation prevents apoptosis through the phosphoinositide 3-kinase pathway. Consequently, the ischemic brain might have been rendered as a nurturing microenvironment characterized by robust angiogenesis and diminished microglial/astrocytic proliferation, resulting in the reduction of infarct volumes and behavioral recovery. Electric stimulation is a novel and potent therapeutic tool for cerebral ischemia. (Stroke. 2009;40:e598-e605.)

Key Words: angiogenesis ★ apoptosis ★ neurotrophic factor ★ regenerative therapy ★ stroke

Electric stimulation therapy has been used in the clinic for various diseases of the central nervous system, including epilepsy, central pain, and psychological disorders like schizophrenia and depression. In particular, deep brain stimulation of the subthalamic nucleus ameliorates motor symptoms of patients with Parkinson disease. For cerebral ischemia, electric stimulation to the affected cortex, sympathoexcitatory dorsal periaqueductal gray matter, spinal cord, contralateral forepaw, muscle of posterior limbs, or epidural space of the motor cortex has been shown to exert functional recovery. In these studies, electric stimulation was thought to participate in the enhancement of synaptic plasticity, suppression of aberrant cell excitation, and facilitation of rehabilitative therapeutic effect. However, there are no reports demonstrating the direct neuroprotective effects of electric stimulation, except for a recent study by Zhou and colleagues whereby electric stimulation of the fastigial nucleus of the cerebellum afforded neuroprotection through the prevention of mitochondrial dysfunction.

Phosphoinositide 3-kinase (PI3K)/Akt signaling pathway has been implicated to play a central role in regulating the activity of many transcriptional factors. PI3K can phosphorylate inositol lipids producing phosphatidylinositol 3, 4, 5-trisphosphate, which, together with its major downstream phosphorylated targets, the serine–threonine kinase Akt, modulates the signal transduction of cell growth.
Conversely, Akt is activated by growth factors such as insulin or platelet-derived growth factor and involved in cell proliferation.\textsuperscript{5,6} Akt suppresses the release of cytochrome c from mitochondria to cytoplasm, which in turn prevents downstream caspase activation.\textsuperscript{7,8} Additionally, Akt is known to be phosphorylated after cerebral ischemia and exerts antiapoptotic effects through PI3K.\textsuperscript{9,10} thus indicating that the control of Akt is a good target for stroke therapy.

In this study, we characterized the neuroprotective properties of electric cortical stimulation in an acute stroke rat model with emphasis on elucidating the underlying mechanisms, including antiapoptotic effects through the phosphorylation of Akt as well as anti-inflammatory and angiogenic pathways.

Materials and Methods

Subjects
Adult male Wistar rats (Charles River, Kanagawa, Japan; \(n=107\)) weighing 200 to 250 g at the beginning of the experiment, according to approved guidelines of the Institutional Animal Care and Use Committee of Okayama University. They were singly housed per cage in a temperature- and humidity-controlled room, maintained on a 12-hour light/dark cycle, with free access to food and water.

Transient Middle Cerebral Artery Occlusion Procedure
Middle cerebral artery occlusion (MCAO) was carried out according to the intraluminal suture method.\textsuperscript{11} All rats were anesthetized (1.0% halothane in 70% \(\text{N}_2\text{O}\) and 30% \(\text{O}_2\)) and a 4-0 monofilament nylon suture with a silicone-coated tip (Xantopren L blue & ACTIVATOR Universal Liquid; Heraeus Kulzer GmbH & Co KG, Hanau, Germany) was inserted through an arteriotomy in the right common carotid artery. The blood supply to the middle cerebral artery was occluded for 90 minutes, whereas the rats were allowed to move freely in the cage and consequently reopened with anesthesia.

Physiological parameters (eg, blood gas values, blood glucose concentration, \(\text{pH}\)) before, during, and after MCAO surgery did not significantly differ across all groups of stroke rats.

Electric Stimulation
At 1 hour after reperfusion, the rats were deeply anesthetized with pentobarbital (35 mg/kg, intraperitoneally) and transplant with twisted bipolar electrodes on the right frontal epidural space, which were located 4 to 4.5 mm lateral from the bregma, with the electrode placement corresponding to the expected cortical surface overlying the ischemic boundary of the cortex (unpublished data using MRI). The electrode was connected to an implantable pulse generator unit (ISE1000SA; UNIMEC Co, Ltd) and electronic stimulator (SEN-7203; NICH KOHDE), which was embedded in the skull with dental cement. Rats were continuously stimulated for 3 days or 1 week after electrode implantation. Stimulating pulses were square-wave pulses and preset at the duration of 1 ms constant current. The parameter of stimulating pulses was adjusted to a variety of electric current (0, 100, 200 \(\mu\)A) and frequency (0, 2, 10, 50 Hz). After the 1-week stimulation, the electric stimulation was discontinued. No epilepsy was induced by electric stimulation.

Measurement of Cerebral Blood Flow
Cortical cerebral blood flow at the right parietal cortex was measured before and at 1 and 2 days after MCAO by using a laser Doppler microvascular perfusion monitor (Laser Flowmeter ALF21; Advance Company, Ltd, Tokyo, Japan). The stainless steel probe (diameter: 0.9 mm) was placed on the skull at 2 mm posterior and 5 mm lateral to the bregma, as we performed in the previous reports.\textsuperscript{12}

Behavioral Assessments: Limb Placement Test
At 30 minutes, 3 days, and 1 week after reperfusion, limb placement testing (LPT) was performed as a behavioral assessment of stroke-induced deficits. LPT consisted of 8 trials to assess the degree of hemiparesis in stroke rats by evaluating the ability to place their limbs on the top or edge of a countertop. For each trial, animals received a score of 0, 1, or 2 (0, unable to place limbs; 1, able to place limbs, but partially and/or taking time, ie, over 2 seconds; 2, able to place limbs immediately and correctly) within a nonischemic area able to achieve a maximum LPT score of 16. Rats with an LPT score of \(\leq 4\) at 30 minutes after reperfusion were used for subsequent experiments because they were appropriate for the ischemic model.\textsuperscript{13}

Measurement of the Infarct Volumes
Rats were euthanized under deep anesthesia using pentobarbital before saline perfusion and brains were quickly removed at 3 days after MCAO for 2,3,5-triphenyltetrazolium chloride staining (\(n=24\)). Thereafter, 6 serial coronal sections of 2-mm thickness were prepared. Brain slices were incubated in a 0.2% solution of 2,3,5-triphenyltetrazolium chloride (Kanto Chemical Co) in phosphate-buffered saline (PBS) at 37°C for 30 minutes and fixed by immersion in 4% buffered formaldehyde solution. The normal area of brain was stained dark red based on intact mitochondrial function, whereas infarct area remained unstained. Each brain slice was scanned by a color flatbed scanner, and infarct area was measured using a computerized image analysis (Image J; National Institutes of Health, Bethesda, Md.). The infarct volumes were calculated as described previously\textsuperscript{14} incorporating edema correction.

Immunohistochemical Investigations
Rats were euthanized with overdosed pentobarbital at 1 week after MCAO for immunohistochemical investigations (\(n=39\)). The rats were perfused transcardially with 200 mL of cold PBS and 200 mL of 4% paraformaldehyde in PBS. Brains were removed and postfixed in the same fixative overnight at 4°C with the subsequent replace- ment with 30% sucrose in PBS for 72 hours. The brains were coronally sectioned at a thickness of 14 \(\mu\)m. Sections were washed 3 times for 5 minutes in PBS. For laminin, Iba-1, glial fibrillary acidic protein (GFAP) staining, sections were incubated overnight at 4°C with each primary antibody and washed 3 times in PBS. The following primary antibodies (final dilution and source) were used: rabbit anti-GFAP (astrocyte marker, 1:1000; Dako Denmark AS), rabbit anti-Iba1 (microglial marker, 1:200; Wako Pure Chemical Industries, Osaka, Japan), and rabbit antilaminin (vascular endothelial marker, 1:500; DAKO). Afterward, sections were incubated with corresponding Cy3-conjugated secondary antibodies (1:1000; Jackson ImmunoResearch Laboratory).

To explore the involvement of apoptosis in this study, a modified method for terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL; Roche) was performed according to the manufacturer’s instructions with subsequent enhancement using an ABC kit and dianinobenzidine staining and finally lightly counterstained with hematoxylin using rats euthanized at 3 days after MCAO. Additionally, to reveal the involvement of phosphorylated Akt, sections were incubated with normal goat serum and reacted with rabbit polyclonal antiphospho-Akt (serine-473) antibody (1:100; Cell Signaling) followed by enhancement using an ABC kit, dianinobenzidine, and hematoxylin. In all immunohistochemical investigations, control studies included exclusion of primary antibody substituted with 10% normal horse serum in PBS. No immunoreactivity was observed in the controls.

Intraventricular Injection of PI3K Inhibitor
To confirm how the neuroprotective effects of electric stimulation might be affected if the PI3K pathway was initially blocked, a PI3K inhibitor, LY294002 (2-[4-morpholino]-8-phenyl-4H-1benzopyran-4-one; Cell Signaling) was intraventricularly administered to rats with or without electric stimulation after cerebral ischemia with subsequent behavioral and histological investigations (\(n=25\)). The
scalloping was counted to evaluate glial reaction. For the analyses of ischemic penumbra, 2 randomly selective areas corresponding to the ischemic cortex and striatum were used as shown in Figure 4. TUNEL and phosphorylated Akt-positive cells in the ischemic penumbra were evaluated using a Zeiss Axiohot microscope. In each evaluation, 6 coronal sections every 336 µm were collected from each rat and cell counts of TUNEL and phosphorylated Akt-positive cells performed. Six representative regions of interest were randomly selected per each section using 6 sections per rat.

### Statistical Analysis

Data are presented as the mean±SE (LPT: score; infarct volumes: percent to the intact hemisphere; laminin-positive structures: ratio to the intact side; GFAP or Iba-1-positive cells: the average of cell counts in randomly selected 4 sections; Western blotting for neurotrophic factors: percent to the integration value of β-actin, Western blotting for phosphorylated Akt: percent to the integration value of Akt). Data were evaluated statistically using Mann–Whitney U test, except for data in experiments using LY294002, using one-way analysis of variance, followed by post hoc Scheffe’s test. Statistical significance was preset at P<0.05.

### Results

#### Electric Stimulation at Suitable Conditions Ameliorates Behavioral Impairment Reduces Infarct Volumes and Increases Cerebral Blood Flow

Initially, we explored therapeutically active regimens for electric stimulation to promote neuroprotection in ischemic brains. Rats received electric stimulation in several conditions with various currents (0, 100, 200 µA) and frequency (0, 2, 10, 50 Hz) for 3 days. Rats receiving electric stimulation of low frequency (2 and 10 Hz at 100 µA) exhibited significant attenuation of behavioral deficit at 3 days and 1 week post-MCAO and reduction of infarct volumes at 3 days post-MCAO (Table; LPT [3 days]: 7.6±3.2 and 8.0±2.7; LPT [1 week]: 11.8±2.2 and 11.1±3.2; infarct volumes: 21.3%±1.4 and 21.8%±6.9% relative to the intact side, for 2 and 10 Hz, respectively) compared with those of control rats and rats with stimulation of higher frequency (ie, 50 Hz; LPT [3 days]: 3.9±1.5 and 4.4±1.7, LPT [1 week]: 6.3±2.7 and 6.2±0.9 for 0 and 50 Hz, respectively; infarct volumes: 28.0%±1.5% and 30.5%±8.7% for 0 and 50 Hz, respectively). The therapeutic effects of electric stimulation at 2 and 10 Hz did not differ, although stimulation at 2 Hz displayed a trend of more potent behavioral and histological benefits than 10 Hz. After confirmation of appropriate frequency, the optimal current was explored. The behavioral amelioration and reduction of infarct volumes were not significantly altered by manipulating the current (LPT [1 week]: 11.8±2.2 and 10.3±3.8; infarct volumes: 21.3±1.4 and 23.9±8.2 for 100 and 200 µA, respectively). The behavioral and histological results for animals that received 0 µA and 0 Hz are the same data as described previously (no current=control). Rats with stimulation at 0, 100 and 200 µA had no damage at the cortex. Based on these results, we decided electric stimulation at 100 µA and 2 Hz was the optimal therapeutic condition for exerting neuroprotection in ischemic stroke and used this setting in subsequent experiments. The percentages of the cerebral blood flow of rats with electric stimulation significantly increased at 2 days after MCAO (88.1%±14.7%) compared with those of rats without electric stimulation.

### Morphological Analyses

Laminin, Iba-1 and GFAP-positive areas in the ischemic penumbra of both cerebral cortex and striatum were examined using a Zeiss LSM510 confocal microscope. To examine angiogenesis, the areas occupied by the laminin-immunopositive structures were calculated in the sections using Image J with 6 representative regions of interest per each section.16 The number of Iba-1 and GFAP-positive cells was counted to evaluate glial reaction. For the analyses of ischemic penumbra, 2 randomly selective areas corresponding to the ischemic cortex and striatum were used as shown in Figure 4. TUNEL and phosphorylated Akt-positive cells in the ischemic penumbra were evaluated using a Zeiss Axiohot microscope. In each evaluation, 6 coronal sections every 336 µm were collected from each rat and cell counts of TUNEL and phosphorylated Akt-positive cells performed. Six representative regions of interest were randomly selected per each section using 6 sections per rat.

### Western Blot Analysis

We next explored whether angiogenic/neurotrophic factors and phospho-Akt were up- or downregulated by electric stimulation. For protein assay, fresh brains from a new cohort of stroke rats with or without electric stimulation (n=6 per group) were quickly removed without electric stimulation (n=6 per group) were quickly removed immediately after decapitation using overdosed pentobarbital (200 mg/kg intraperitoneally) at 1 week after the initiation of electric stimulation. Brains were sliced at a thickness of 2 mm. Each pair of brain tissues of the cortex and striatum per hemisphere was punched out using Harris Uni-Core (2-mm hole; TLED PELLA, Inc, CA). Brain tissues were then suspended in the lysis buffer (Cellytic MT; Sigma) and centrifuged at 15 000 rpm for 10 minutes at 4°C twice to remove cell debris, nuclei, and large particulates and obtain the supernatant. Two volumes of blue/orange 6× Loading Dye (Promega) were added and boiled at 95°C for 4 minutes. Samples were subjected to electrophoresis in a 7.5% sodium dodecyl sulfate–polyacrylamide gel and transferred electrophoretically to a Hybond-P pure nitrocellulose membrane (Amerham Pharmacia Biotech, Buckinghamshire, UK). The membranes were probed at room temperature for 12 hours with mouse anti-vascular endothelial growth factor (VEGF) antibody (1:1000, #555036; BD Pharmingen), rabbit anti-glial cell line-derived neurotrophic factor (GDNF) antibody (1:500, #sc-328; Santa Cruz, CA), rabbit anti-brain-derived neurotrophic factor (BDNF) antibody (1:500, #546; Santa Cruz), rabbit antiphosphorylated Akt antibody (1:500, #9271; Cell Signaling), rabbit anti-Akt antibody (1:1000, #9272; Cell Signaling), and antimouse anti-β-actin (1:2000, A5441; Sigma) as primary antibodies followed by exposure to horseradish peroxidase-conjugated secondary antibodies (1:2000, antimouse IgG; 1:2000, antirabbit IgG; Amerham Pharmacia Biotech; 1:2000) for 1 hour. Signal development was achieved using an ECL plus Detection kit (Amerham Biosciences).14 The relative densities and areas of the bands were analyzed with Image J software, the numeric integration value (density×area) was calculated, and finally corrected using the value of β-actin.15

### Table. LPT Scores and the Infarct Volumes of Rats Receiving Electrical Stimulation With Several Parameters

<table>
<thead>
<tr>
<th>Value</th>
<th>0</th>
<th>2</th>
<th>10</th>
<th>50</th>
</tr>
</thead>
<tbody>
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<td><strong>LPT (Hz)</strong></td>
<td>100</td>
<td>63.2</td>
<td>28.0</td>
<td>200</td>
</tr>
<tr>
<td><strong>Infarct Volumes (%)</strong></td>
<td>3.9±1.5</td>
<td>7.6±3.2</td>
<td>8.2±2.2</td>
<td>4.4±1.7</td>
</tr>
<tr>
<td><strong>LPT (1 week)</strong></td>
<td>6.3±2.7</td>
<td>11.8±2.2</td>
<td>11.1±3.2</td>
<td>6.2±0.9</td>
</tr>
<tr>
<td><strong>Infarct Volumes (%)</strong></td>
<td>28.0±1.5</td>
<td>21.3±1.4</td>
<td>21.8±6.9</td>
<td>30.5±8.7</td>
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**Table:** The table demonstrates LPT scores (3 days and 1 week after MCAO) and the percentages of infarct volumes relative to the intact side of rats receiving electric stimulation with several parameters. Initially, 0, 2, 10, and 50 Hz were used with current at 100 µA. Then exploration using 200 µA was performed. Upper: LPT score at 3 days; middle: LPT score at 1 week; lower: the percentages of infarct volumes at 3 days post-MCAO.

### Materials and Methods

#### Animal Care

Rats were cared for in accordance with the institutional guidelines for animal experiment and approved by the Animal Experiment Ethic Committee of the Fourth Military Medical University. Rats were anesthetized with an ip injection of pentobarbital (200 mg/kg) and perfused with 0.9% saline followed by 4% paraformaldehyde. Cerebral cortex and striatum were dissected immediately and treated as above.
Although there were no significant differences between the 2 groups when cerebral blood flow was measured during MCAO or 1 day after MCAO (during MCAO: 26.2% ± 5.1% and 24.4% ± 4.6%; at 1 day after MCAO: 72.4% ± 10.2 and 74.5% ± 7.4% in rats without and with electric stimulation, respectively).

**Figure 1.** Angiogenic and anti-inflammatory effects of electric stimulation. Laminin staining (A–D) revealed that angiogenesis was enhanced by electric stimulation in the ischemic cortex (C–D) compared with the nonstimulated cortex (B) and the intact cortex (A). Quantitative analyses shown in graph (M) reveal significant differences in the percentages of laminin-positive structures in the cortex, but not in the striatum between the electric stimulation and control groups. Iba-1 staining (E–H) revealed that microglial proliferation was suppressed by electric stimulation in the ischemic cortex (G) compared with the nonstimulated cortex (F, H): note that Iba-1-positive cells were hardly seen in the intact cortex (E). Quantitative analyses (N) also showed significant reduction of the number of Iba-1-positive cells in the cortex, but not in the striatum between the electric stimulation and control groups. GFAP staining (I–L) revealed that astrocytic proliferation was suppressed by electric stimulation in the ischemic cortex (K) compared with the nonstimulated cortex (I, L); also note that GFAP-positive cells were hardly seen in the intact cortex (I). Quantitative assessment (O) similarly revealed significant reduction of the number of GFAP-positive cells in the cortex, but not in the striatum between the stimulation and control groups. Data are shown as the mean percentages (laminin) or the cell number (Iba-1 and GFAP) ± SE. *P < 0.05 versus cortex of the control rats. Scale bar: 100 μm (A–C, E–G, I–K), 25 μm (D, H, L).

Electric stimulation increased laminin-positive vessels in the ischemic penumbra of cerebral cortex compared with that of rats without electric stimulation (referred to control rats hereafter; Figure 1). The area occupied by laminin-positive vessels in the ischemic penumbra of the cerebral cortex with electric stimulation was significantly larger (13.6 ± 1.3) than the control (9.0 ± 0.8). GFAP staining revealed a widespread infiltration of amoeboid, phagocytic, and spherical fully activated astrocytes in the ischemic penumbra of the control rats, which was only observed sporadically and with morphological features characteristic of small astrocytes in the ischemic penumbra of rats that received electric stimulation (Figure 1). The number of GFAP-positive cells in the ischemic penumbra of the cerebral cortex of rats with electric stimulation was significantly smaller (332 ± 63 cells/0.2 mm³) than that of the control rats (587 ± 53 cells/high-power field). Similarly, Iba1 staining revealed rampant proliferation and activation of microglia in the ischemic penumbra in the cerebral cortex of the control rats, whereas microglial activity appeared suppressed in rats receiving electric stimulation. The number of Iba-1-positive cells in the ischemic penumbra of rats receiving electric stimulation was significantly smaller (79.7 ± 21 cells/0.2 mm³) than the control (173 ± 49 cells/0.2 mm³). Interestingly, laminin, GFAP, and Iba-1 staining revealed significant treatment effects in the ischemic penumbra of the cerebral cortex and similar trends, but not statisti-
cally significant, in the striatum (laminin: 15.2±1.2 and 11.6±1.0, \( P=0.089 \); GFAP: 524±47 and 611±116, \( P=0.16 \); Iba-1: 191±48 and 218±33, \( P=0.31 \) for rats with and without electric stimulation, respectively) might be in the same direction as those in the cortex.

**Electric Stimulation Triggers Antiapoptotic Effects Through Akt Phosphorylation**

TUNEL staining revealed significantly few TUNEL-positive cells in the ischemic cortex of rats receiving electric stimulation at 3 days post-MCAO (121±11 cells/mm²) compared with those of control rats (274±16 cells/mm²; Figure 2). However, there was no significant difference in the TUNEL staining of the ischemic striatum between 2 groups (electric stimulation: 225±17, control: 276±21 cells/mm²). In our desire to decipher the antiapoptotic pathway affected by the electric stimulation, we investigated Akt phosphorylation. Immunohistochemical analyses revealed upregulation of phosphorylated Akt not only in the cortex, but in the striatum of rats with electric stimulation (cortex: 681±66 cells/mm², striatum: 478±33 cells/mm²) compared with that of control rats (cortex: 284±39, striatum: 253±31 cells/mm²). Similarly, Western blotting assays revealed that phosphorylated Akt was significantly upregulated both in the cortex (37.7%±0.8% to the level of Akt) and striatum (34.7%±0.9%) of rats with stimulation compared with those of control rats (cortex: 6.0%±0.2%, striatum: 7.7%±0.3%).

**Neutralizing Effects of LY294002 on Neuroprotective Effects Induced by Electric Stimulation**

With the recognition that the antiapoptotic effects of electric stimulation involved phosphorylated Akt, we then pharmacologically examined whether the neuroprotection entailed the PI3K/Akt signaling pathway by pretreating stroke rats with the PI3K inhibitor LY294002 (Figure 3). Western blotting assay revealed LY294002 significantly suppressed phosphorylation of Akt. Moreover, LPT scores of rats exposed to electric stimulation and LY294002 infusion worsened (6.6±0.3) compared with those of rats receiving electric stimulation and vehicle treatment (12.6±0.3). LY294002 infusion alone did not alter LPT score compared with the control (LY294002 infusion only: 6.7±0.3, control: 8.2±0.2, \( F_3, 21=17.2, P<0.001, P<0.05 \). 2,3,5-Triphenyltetrazolium chloride staining showed that infarct volumes of rats receiving LY294002 infusion and electric stimulation were significantly larger (27.0%±1.2% relative to the intact hemisphere) than those of rats that underwent electric stimulation without LY294002 infusion (13.1%±0.2%). LY294002 infusion alone did not signifi-
cantly affect infarct volumes compared with control rats (LY294002 infusion only: 26.9%/H_11006 1.2%, control: 25.3%/H_11006 1.5%, F_3, 21 = 35.3, P < 0.001, P < 0.05).

Expression of Trophic Factors in the Ischemic Brain After Electric Stimulation

Because downstream neuroprotective mechanisms implicate crosstalk between the PI3K/Akt signaling pathway and growth factors in the brain, we pursued the notion that electric stimulation upregulated the expression of trophic factors resulting in antiapoptotic effects accompanied by enhanced angiogenesis and suppressed inflammation in stroke rats (Figure 4). BDNF, GDNF, and VEGF were all upregulated in the ischemic penumbra of both cerebral cortex and striatum of rats receiving electric stimulation (BDNF: 56.7%/H_11006 1.1% and 41.1%/H_11006 3.0%, GDNF: 159%/H_11006 10% and 193%/H_11006 18%, and VEGF: 91%/H_11006 8.7% and 66.5%/H_11006 5.8% relative to the integral value of \beta-actin band in the cerebral cortex and striatum) compared with those of control rats (BDNF: 35.8%/H_11006 5.2% and 22.0%/H_11006 2.8%, GDNF: 125%/H_11006 5.1% and 113%/H_11006 20%, and VEGF: 27.8%/H_11006 7.0% and 34.4%/H_11006 3.4% relative to the integral value of \beta-actin band in the cerebral cortex and striatum).

Discussion

The present study showed that electric cortical stimulation exerted neuroprotective effects on transient MCAO stroke rats in the acute stage as reflected by amelioration of behavioral deficits, attenuation in both necrotic and apoptotic cell death, and blockade of microglial activation. Here, we observed that electric stimulation upregulated neurotrophic factors (BDNF, GDNF) and an angiogenic factor (VEGF) in tandem with suppression of apoptosis through the phosphorylation of Akt.

Electric Stimulation for Cerebral Ischemia

A recent small pilot clinical study reported that patients with stroke receiving cortical stimulations displayed a significant improvement in the function of their affected upper limb. Similarly, spinal cord stimulation produced therapeutic benefits in patients with brain injury by increasing cerebral blood flow. These studies, coupled with accumulating preclinical experiments, suggest that electric stimulation for treating cerebral ischemia is likely poised to have direct clinical applications.

The therapeutic regimens for electric stimulation vary with the target disease indication. The effective conditions of deep brain stimulation, clinically used for patients with Parkinson disease, are, for example, 130 Hz, 20 to 30 \mu A (2V), which suppressed the activity of affected neurons. Similarly, the high frequency stimulation at 100 Hz in the bilateral central thalamus was shown to significantly improve the behavioral scores of patients with severe traumatic brain injury at the chronic stage. The low frequency (2 Hz) of our electric stimulation might provide a unique treatment approach in that the subtle stimulation may facilitate, rather than diminish, neuronal activity, which is inherent with high-frequency electric stimulations, as demonstrated in a mouse model of Parkinson disease. Of note, high-frequency electric stimulation has regularly used an adjunct rehabilitative therapy that

Figure 3. Neutralizing effects of LY294002 on neuroprotective effects of electric stimulation. The infarct volumes of rats receiving electric stimulation (B) were significantly decreased compared with those of control rats (A) and ischemic rats that received LY294002 alone (C). In contrast, the neuroprotective effects of electric stimulation were blocked by intraventricular administration of LY294002 (D). Quantification of infarct volumes is shown in E. Data correspond to mean percentages ± SE. *P < 0.05 versus all other groups.
likely aided in improved outcome after stroke. Thus, the present low-frequency electric stimulation represents a paradigm shift in affording neuroprotection. To this end, the constant exposure to low-frequency electric stimulation appears as an alternative to bursts of high-frequency electric stimulation. A 1-week electric stimulation to the cortex significantly increased the action potentials of the damaged neurons and reduced the required current to elicit motion with subsequent recovery of function in stroke rats. Accordingly, the extensive application of low-frequency electric stimulation over 1 week appears sufficient to achieve functional recovery.

Antiapoptotic Effects of Electric Stimulation Through the Phosphorylation of Akt

Antiapoptotic effects of electric stimulation were previously demonstrated using the degenerated auditory nerve, which required the propagation of action potentials with complete blockade by tetrodotoxin. Using the myocardium, similar antiapoptotic responses were elicited with 5 Hz and further implicated Akt activation. LY294002, a PI3K inhibitor used in the present study, increased TUNEL-positive apoptotic cells in their experiment, thus suggesting the phosphorylation of Akt is closely associated with the antiapoptotic effects of electric stimulation. For the first time in a cerebral ischemia model, the present study revealed that electric stimulation enhanced phosphorylated Akt concomitant with reduced TUNEL apoptotic cell marker.

Anti-Inflammatory and Angiogenic Effects of Electrical Stimulation

Inflammation may be a process of self-protection by the brain against invasion of injurious prodeath cells from the periphery. Microglial/astrocytic hyperactivity at the acute stage of stroke usually leads to secondary damage. The suppression of microglial/astrocytic activation in stroke rats was demonstrated to reduce infarct volumes, but did not alter microglial proliferation. In parallel, examination of tissues adhered to the electrodes used for deep brain stimulation in patients with Parkinson disease exhibited no suppression of microglial activation. The discrepant results in microglial activation after electric stimulation may be due to the disease pathophysiology (Parkinson disease versus cerebral ischemia), duration of the electric stimulation (several years versus 1 month), or species (human versus rat).

Along the same vein of affording neuroprotection through suppression of microglial activation, trophic factors have been shown to regulate this anti-inflammatory response. In previous studies, GDNF suppressed microglial activation, whereas VEGF activated the microglial proliferation, and upregulation of BDNF-immunoreactive microglial cells seems to confer neuroprotection at the sites of excitotoxin-induced neurodegeneration. In the end, a homeostatic balance in microglial activation and suppression by a cocktail of counteracting trophic factors primed by electric stimulation may translate into neuroprotection. The synergistic effects of trophic factors upregulated by electric stimulation likely led to modulation of microglial/astrocytic activation resulting in anti-inflammatory effects, which directly rendered neuroprotection and/or in part contributed to the reduction in infarct volumes.

As described, Akt phosphorylation was activated by electric stimulation. Of interest, such electric stimulation-mediated Akt activation was found to aid in angiogenesis of
cultured cells by upregulation of VEGF as well as to enhance angiogenesis after MCAO in rats. Together, these results suggest that a myriad of neuroprotective mechanisms ensues after electric stimulation primarily targeting the PI3K/Akt signaling pathway.

**Summary**

The present study demonstrated that cortical stimulation afforded neuroprotection in an ischemic stroke rat model. Here, we invoked the neuroprotection cascade along the anti-apoptotic pathway through the phosphorylation of Akt leading to anti-inflammatory and angiogenic effects with equally compelling evidence implicating the contribution of trophic factor upregulation. This is the first report to reveal a multipronged neuroprotective property of electric stimulation. Additional studies are warranted to assess safety and long-term effects of electric stimulation in ischemic stroke.

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**Disclosures**

None.

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