The Phosphodiesterase Inhibitor Rolipram Promotes Survival of Newborn Hippocampal Neurons After Ischemia

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Background and Purpose—Brain ischemia stimulates neurogenesis. However, newborn neurons show a progressive decrease in number over time. Under normal conditions, the cAMP–cAMP responsive element binding protein (CREB) pathway regulates the survival of newborn neurons. Constitutive activation of CREB after brain ischemia also stimulates hippocampal neurogenesis. Thus, activation of cAMP-CREB signaling may provide a promising strategy for enhancing the survival of newborn neurons. We examined whether treatment of mice with the phosphodiesterase-4 inhibitor rolipram enhances hippocampal neurogenesis after ischemia.

Methods—Both common carotid arteries in mice were occluded for 12 minutes. Bromodeoxyuridine (BrdU) was used to label proliferating cells. Mice were perfused transcardially with 4% paraformaldehyde, and immunohistochemistry was performed. To evaluate the role of CREB in the survival of newborn neurons after ischemia, intrahippocampal injection of a CRE-decoy oligonucleotide was delivered for 1 week. We examined whether the activation of cAMP-CREB signaling by rolipram enhanced the proliferation and survival of newborn neurons.

Results—Phospho-CREB immunostaining was markedly upregulated in immature neurons, decreasing to low levels in mature neurons. The number of BrdU-positive cells 30 days after ischemia was significantly less in the CRE-decoy treatment group than in the vehicle group. Rolipram enhanced the proliferation of newborn cells under physiologic conditions but not under ischemic conditions. Rolipram significantly increased the survival of nascent BrdU-positive neurons, accompanied by an enhancement of phospho-CREB staining and decreased newborn cell death after ischemia.

Conclusions—CREB phosphorylation regulates the survival of newborn neurons after ischemia. Chronic pharmacological activation of cAMP-CREB signaling may be therapeutically useful for the enhancement of neurogenesis after ischemia. (Stroke. 2007;38:1597-1605.)

Key Words: CREB ■ hippocampus ■ neurogenesis ■ ischemia

Neurogenesis continues throughout adulthood in the subgranular zone (SGZ) of the hippocampus and in the subventricular zone.1,2 Brain ischemia also enhances neurogenesis in the hippocampus and also induces the migration of neuroblasts into lesions of nonneurogenic areas such as the striatum.3 However, only a small fraction of these newborn neurons derived from proliferating progenitors survive.2,3

The transcription factor, cAMP responsive element binding protein (CREB), mediates diverse responses in the nervous system, such as learning, memory, neuronal plasticity, and cell survival.4 In addition to its function in mature neurons, CREB regulates cell proliferation, differentiation, and survival in the developing brain. Phosphorylation of CREB at Ser 133 is crucial for CREB-dependent transcription.5

Under normal conditions, the cAMP-CREB pathway regulates multiple aspects of adult hippocampal neurogenesis.6,7 Activation of CREB by expression of constitutively active VP16-CREB after brain ischemia also stimulates hippocampal neurogenesis.8 A therapeutic pharmacological approach to increase cAMP and activate cAMP-CREB signaling involves inhibition of the degradation enzyme phosphodiesterase (PDE). Among the PDE subfamily, PDE4 represents 70% to 80% of PDE activity in neuronal tissue.9 cAMP-CREB signaling in hippocampal neurogenesis is very important. However, the effect of pharmacological activation of the cAMP-CREB signaling pathway after ischemia has not been elucidated. In this study, we sought to determine whether pharmacological activation of this pathway by the PDE4 inhibitor rolipram enhances survival of newborn neurons in the hippocampal dentate gyrus under ischemic conditions.

Materials and Methods

Animals
Adult male C57BL/6 mice (11 to 12 weeks old) were used in this study. The experimental protocol was approved by the institutional
animal care and use committee of Osaka University Graduate School of Medicine. They were fed standard laboratory chow and had access to water ad libitum before and after all procedures. Animal care was given according to the guidelines of the animal center of Osaka University Graduate School of Medicine.

Bromodeoxyuridine Labeling Protocols and Immunohistochemistry
Bromodeoxyuridine (BrDU; Roche Diagnostics, Indianapolis, Ind), a thymidine analog, was used to label proliferating cells. To quantify BrDU-positive cells and evaluate the phenotype of postmitotic cells, mice were injected intraperitoneally with BrDU (50 mg/kg) 4 times every 2 hours for 6 hours and killed at 1, 3, 7, 14, or 30 days after BrDU administration. Mice were killed under deep pentobarbital anesthesia and perfused transcardially with 4% paraformaldehyde, and the brains were removed and fixed in 4% paraformaldehyde at 4°C.

The protocol for BrDU immunohistochemistry was as described previously. In brief, sections were treated with 50% formamide and 2X saline-sodium citrate buffer and then incubated in 2N HCl. After a wash, sections were incubated with rat monoclonal anti-BrDU antibody (1:100, Harlan Sera-Laboratory, Loughborough, UK) at 4°C overnight. After a wash, sections were incubated with biotinylated secondary antibody, washed, and then incubated with streptavidin-biotin-peroxidase complex (Vector Laboratories, Burlingame, Calif). Sections were reacted with 0.05% 3'-diaminobenzidine in the presence of 0.01% H2O2.

For double immunofluorescence, free-floating sections (40 μm) were incubated with primary antibody diluted in Tris-buffered saline/0.1% Triton X-100 containing 1.5% normal serum at 4°C overnight. The following primary antibodies were used for immunofluorescence: rat monoclonal anti-BrDU antibody (1:100, Harlan Sera-Labos), mouse monoclonal anti-BrDU antibody (1:200, Amersham, Piscataway, NJ), rabbit polyclonal anti-phospho-CREB (pCREB) antibody (1:200, Upstate Biotechnology, Lake Placid, NY), rabbit polyclonal anti-CREB antibody (1:200, Cell Signaling, Beverly, Mass), mouse monoclonal anti-NeuN antibody (1:200, Chemicon, Temecula, Calif), rabbit polyclonal anti-glial fibrillary acidic protein antibody (1:200, Sigma-Aldrich, St. Louis, Mo), goat polyclonal anti-doublecortin (DCX) antibody (1:100, Santa Cruz Biotechnology, Santa Cruz, Calif), rat monoclonal anti-Musashi-1 (Msi-1) antibody clone 14H11 (1:200, and mouse monoclonal polysialylated neural cell adhesion molecule (PSA-NCAM) antibody (1:200, Pharmingen, San Jose, Calif).

For double labeling of BrDU and cell markers (NeuN for mature neurons; glial fibrillary acidic protein for astrocytes; DCX and PSA-NCAM for migrating neuroblasts and immature neurons; and Msi-1 for neuronal progenitors, neuronal stem cells, and astrocytes), sections were subjected to DNA denaturation and incubated with the appropriate anti-BrDU antibody plus antibody to 1 of the aforementioned cell markers at 4°C overnight. Sections were then incubated with an appropriate anti-IgG or anti-IgM secondary donkey antibody conjugated to fluorescein isothiocyanate (FITC) or rhodamine (1:200, Chemicon) for 90 minutes at room temperature. After a rinse in Tris-buffered saline, sections were mounted with Vectorshield (Vector Laboratories), and staining was visualized with a confocal microscopy system (LSM-510, Zeiss, Oberkochen, Germany).

Transient Forebrain Ischemia
General anesthesia was maintained with 1% halothane by means of an open facemask. A polyacrylamide column for measurement of cortical microperfusion by laser Doppler flowmetry (Advanced Laser Flowmetry) was attached to the skull, 3 mm lateral to the bregma on the right side, with dental cement. Body and skull temperatures were monitored and maintained at 36.5°C to 37.5°C with a heat lamp and mat. Both common carotid arteries were occluded for 12 minutes with microaneurysm clips and then reperfused. As described previously, only mice that showed <13% of baseline control microperfusion during the first minute of occlusion were used in subsequent experiments. We injected BrDU (50 mg/kg, IP) 9 days after ischemia, as previously reported. Thereafter, mice subjected to ischemia were processed under the same schedule as those under normal conditions.

CRE-Decoy Oligonucleotide Administration
To evaluate the importance of CREB for the survival of newborn neurons after ischemia, intrahippocampal injection of a CRE decoy was delivered by means of a miniosmotic pump starting 7 days after BrDU labeling and continuing for 1 week. Treatment with the CRE-cutout oligonucleotide was performed as described previously with some modification. EARCRE-decoy and control oligonucleotides used in this experiment were phosphorothioate oligonucleotides (OligoExpress, Sigma, Tokyo, Japan). Their sequences were as follows: 24-mer CRE-decoy, 5′-TGAGCTTAGCTAGCAGTACG-GTCA-3′ and 24-mer nonsense-sequence control, 5′-CTAGCATTAGCTAGCTAG-3′. Because the CRE cis-element, TGACG-TCAG, is palindromic, it was shown that the CRE-decoy oligonucleotide self-hybridized to form a duplex hairpin and competed with CRE enhancers for binding transcription factors and specifically interfered with CRE-directed transcription in vitro.

Anesthetized mice were placed in a stereotaxic frame, and a mixture (100 μL) consisting of 10 μM oligonucleotide dissolved in N-[1-(2,3-Dioleyloxy)l-N,N,N-trimethylammonium propane methylsulfate (DOTAP) solution was infused unilaterally into the hippocampus with an osmotic pump (1007D, Alza Corp, Mountain View, Calif) attached to a cannula (brain infusion kit II, Alza Corp) implanted at 1.5 mm lateral and 2.0 mm caudal to the bregma to a depth of 2.5 mm from the dura. The CRE decoy was infused 7 days after BrDU labeling for 7 days at a flow rate of 0.5 μL/h. Intrahippocampal injection was confirmed by noting the presence of injection scars on fixed brain slices. For immunohistochemical examination of hippocampal CRE-decoy oligonucleotide distribution, brains were removed 24 hours after FITC-labeled CRE-decoy injection. To quantify the survival of newborn neurons, mice were anesthetized and perfusion-fixed with 4% paraformaldehyde as described earlier. The number of surviving newborn neurons was compared with that at 30 days after BrDU labeling.

Rolipram Treatment
To evaluate the effect of rolipram on the proliferation of newborn cells, normal mice received injections of rolipram daily for 7 consecutive days. Ischemic mice received injections of rolipram (3 mg/kg or vehicle IP) starting 3 days after ischemia and daily for 7 consecutive days. BrDU was given every 2 hours for 6 hours beginning 1.4 mm caudal and 1.9 mm caudal to the bregma. To evaluate the effect of rolipram on the survival of newborn cells, BrDU was given 4 times every 2 hours for 6 hours at 2 hours after the last rolipram injection. Both group of mice were killed 1 or 28 days after the last BrDU injection. Next, to evaluate the effect of rolipram on the survival of newborn neurons, BrDU was given 4 times every 2 hours for 6 hours at 9 days after ischemia. Rolipram (1 or 3 mg/kg or vehicle IP) was administered starting 7 days after BrDU labeling and daily for 21 consecutive days. At 30 days after BrDU labeling, the number of surviving newborn neurons was compared.

TUNEL Staining
To identify cell apoptosis, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) labeling was performed. The brain was removed rapidly en bloc and quickly frozen in liquid N2 vapor. Fourteen-micron-thick sections were cut on a cryostat and postfixed in 1% paraformaldehyde for 10 minutes. The Apoptag fluorescein in situ apoptosis detection kit (S7110, Chemicon, Temecula, Calif) was then applied. For immunofluorescence double labeling of TUNEL signal and BrDU, the TUNEL-fluorescein labeling was performed first, followed by incubation in 2N HCl for 30 minutes at 37°C, followed by application of a rat monoclonal anti-BrDU antibody.

Quantification
To count BrDU-positive cells, 5 sections from the hippocampus were cut every 120 μm beginning 1.4 mm caudal and 1.9 mm caudal to the bregma. In the hippocampus, the granular cell layer (GCL) and SGZ,
defined as a zone 2 cell bodies wide along the border of the GCL and hilus, were considered together for quantification. The mean density of BrdU-positive cells in each mouse was calculated as the number of labeled nuclei divided by the area.

To assess the phenotype of BrdU-positive cell in double immuno-fluorescence, a mean value for each marker was obtained from 10 sections from 5 to 8 mice. Data in the text and figure are described mean±SD. Multiple comparisons were evaluated statistically by ANOVA, followed by Scheffe’s post hoc tests.

**Results**

To assess BrdU-positive newborn cells in the dentate gyrus over time, we determined the number of BrdU-positive cells and the phenotype of postmitotic cells at 1 and 30 days after BrdU administration. Under normal conditions, the number of BrdU-positive cells showed a progressive decrease (1 day, 39.4±8.6/mm²; 30 days, 9.6±3.6/mm²). Under ischemic conditions, the numbers of BrdU-positive cells was 115.5±23.7/mm² at 1 day and 38.7±13.1/mm² at 30 days.

To examine the expression of pCREB in newborn hippocampal neurons, analysis of pCREB staining at various times after BrdU administration was carried out (Figure 1). Only a few BrdU-positive cells in the SGZ and GCL showed pCREB staining 1 day after BrdU administration (Figure 1A). pCREB staining was detected as early as 3 days after BrdU administration and increased in number thereafter. At 14 days after BrdU administration, a majority of BrdU-positive cells showed pCREB staining (Figure 1A). pCREB staining of BrdU-positive cells decreased dramatically by 30 days after BrdU injection. The profile of BrdU-positive cells showing pCREB staining under ischemic conditions over time was similar to that under normal conditions (Figure 1B). Semi-quantitative analysis showed the following with respect to BrdU-plus-pCREB–positive cells in the SGZ and GCL: 0% at 1 day, 35.3±6.6% at 3 days, 70.0±3.1% at 7 days, 81.0±6.6% at 14 days, and 0% at 30 days in the normal group and 12.1±5.2% at 1 day, 52.5±9.0% at 3 days, 77.1±4.7% at 7 days, 80.5±10.4% at 14 days, and 0% at 30 days in the ischemia group (Figure 1C). Also, there was an increase in the total number of pCREB-positive cells at 10 days (9±1 days) or 37 days (9±28 days) after ischemia (control, 433.3±54.9/mm²; 10 days, 638.9±59.2/mm²; 37 days, 553.5±32.0/mm²; Figure 1D).

To identify the phenotype of pCREB-positive cells, we performed double immunolabeling with BrdU antibody and for neuronal progenitor/neuronal stem cell (Msi-1), immature neuron (DCX, PSA-NCAM), or mature neuron...
(NeuN) markers (Figure 2). Msi-1–positive cells in the SGZ and GCL did not stain for pCREB. PSA-NCAM–positive immature neurons showed intense pCREB immunoreactivity (arrows). NeuN-positive mature neurons showed low levels of pCREB staining (Figure 2A). Although only cells within or near the SGZ were positive for pCREB expression (Figures 2A through 2C), most cells throughout the GCL show CREB immunoreactivity (Figure 2D).

To assess the importance of pCREB in immature newborn neurons, inhibition of the CREB-CRE cascade by CRE-decoy oligonucleotide administration was performed. To confirm the distribution of infused CRE-decoy oligonucleotide, brains were removed 24 hours after FITC-labeled CRE-decoy injec-

Figure 2. A, Double-immunofluorescence staining of pCREB/Msi-1, pCREB/PSA-NCAM, and pCREB/NeuN in the SGZ. Msi-1–positive cells did not stain for pCREB. PSA-NCAM–positive immature neurons showed intense pCREB immunoreactivity (arrows). NeuN–positive mature neurons showed low levels of pCREB immunostaining (arrowheads). B, Colocalization of DCX and pCREB immunostaining in the SGZ after ischemia (arrows). C, Higher-magnification views of selected individual z planes and a series through the section (distance is 10 μm). D, CREB immunostaining in the dentate gyrus. Bars=30 μm in A; 20 μm in B, C, and D.
tion. Intense FITC immunoreactivity was observed throughout the pyramidal layer and the dentate gyrus on the infused side but not on the contralateral side, indicating that the FITC–CRE-decoy oligonucleotide did not diffuse contralaterally (Figure 3B). Next, to examine the phenotype of the FITC–CRE-decoy oligonucleotide-positive cells in the dentate gyrus in the hippocampus, double immunofluorescence was performed. Most DCX-positive immature neurons and MAP2-positive mature neurons showed colocalization of FITC immunostaining (Figure 3C, a–c). Also, the infusion of CRE-decoy oligonucleotide decreased the pCREB immunoreactivities throughout the dentate gyrus (Figure 3C, d).

Under normal conditions, the numbers of BrdU-positive cells in the dentate gyrus of control, control oligonucleotide−,
CRE-decoy (ipsilateral)–, and CRE-decoy (contralateral)–treated groups were 11.9 ± 5.3, 10.0 ± 5.0, 6.7 ± 4.9, and 9.6 ± 6.7/mm², respectively (Figure 4C). Under ischemic conditions, mice treated with CRE-decoy oligonucleotide showed a significant decrease in the number of BrdU-positive cells in the dentate gyrus compared with control mice at 30 days after BrdU administration (44.2 ± 23.4/mm² in control mice, 43.2 ± 10.5/mm² in mice treated with control oligonucleotide, 19.8 ± 8.5/mm² in mice treated with CRE-decoy oligonucleotide [ipsilateral], and 40.5 ± 18.4/mm² in CRE-decoy oligonucleotide [contralateral], n=6). Additionally, mice treated with CRE-decoy oligonucleotide showed a significant decrease in the number of NeuN- and BrdU-double-positive cells on the infused side in the SGZ compared with control oligonucleotide–treated mice at 30 days after BrdU administration (10.0 ± 2.3/mm² in the CRE-decoy oligonucleotide–treated mice on the infused side, 25.9 ± 6.0/mm² in the control oligonucleotide–treated mice).

To evaluate the effect of rolipram on the proliferation of newborn cells under normal and ischemic conditions,
rolipram-treated and vehicle-treated mice were killed 1 or 28 days after BrdU administration (Figure 4). Under normal conditions, the number of BrdU-positive cells in the rolipram-treated (3 mg/kg) mice (127.6±30.1/section) was significantly increased over that in the vehicle-treated mice (97.6±11.2/section) 1 day after BrdU administration (Figures 4B and 4C). Because not many newborn cells survived, the total number of BrdU-positive newborn cells in mice treated with rolipram and vehicle decreased proportionally at this time point. However, there was still a significant increase in the number of BrdU-positive cells in rolipram-treated mice relative to vehicle (23.8±4.2/section in the rolipram group, 10.4±3.8/section in the vehicle group; Figures 4B and 4C). In contrast, under ischemic conditions, no significant differences were noted between rolipram-treated (3 mg/kg) mice (274.6±45.4/section) and vehicle-treated mice (252.5±55.4/section) 1 day after BrdU administration (Figures 4B and 4C). At 28 days after BrdU administration, the number of BrdU-positive cells was 115.4±29.2/section in the rolipram-treated mice and 108.2±15.0/section in the vehicle-treated mice.

Next, to evaluate the implication of pCREB expression in immature neurons, we examined the effect of rolipram on the survival of newborn cells. Treatment with 3 mg/kg rolipram from 7 to 28 days after BrdU administration significantly increased the numbers of both BrdU-positive cells (35.1±11.2/mm² in the vehicle mice, 42.2±12.1/mm² in 1 mg/kg rolipram-treated mice, 56.9±18.2/mm² in 3 mg/kg rolipram-treated mice) and BrdU- and NeuN-double-positive newborn cells (18.9±6.7/mm² in the vehicle-treated group, 27.3±7.3/mm² in mice treated with 1 mg/kg rolipram, 31.5±10.0/mm² in mice treated with 3 mg/kg rolipram; Figure 5B). Consistent with these results, daily injection of 3 mg/kg rolipram for 3 weeks significantly increased pCREB staining in DCX-positive immature neurons (55.5±8.2% in the vehicle mice, 75.0±10.4% in 3 mg/kg rolipram-treated mice; Figure 5C). To assess the contribution of apoptotic cell death to the progressive reduction, we used double immunolabeling with anti-BrdU antibody and TUNEL staining. Under ischemic condition, the number of BrdU/TUNEL double-positive cells in the rolipram-treated mice (0.4±0.3/section, n=9) was significantly lower than that in the vehicle group (1.6±0.8/section, n=9).

Discussion

The present findings provide insight into the role of CREB in adult neurogenesis after brain ischemia. Previous studies have shown that running exercise and enriched environment promote the survival of newly generated neurons. An enriched environment has also been shown to enhance pCREB expression in immature neurons of the adult hippocampus.16

Previous experiments have suggested that, under physiologic conditions, cAMP-CREB signaling plays an important role in the dentate gyrus in the hippocampus, and activation of cAMP-CREB signaling influences multiple aspects of neurogenesis, including the proliferation, survival, differentiation, and maturation of newborn neurons.6,7,17 In agreement with reports under physiologic conditions, we found that rolipram treatment enhanced the survival of newborn cells in the hippocampal dentate gyrus. However, after ischemia, in contrast to the enhancing effect of rolipram on the survival of new neurons, rolipram had no significant effect on the proliferation of newborn cells in the hippocampal dentate gyrus. The discrepancy between results under physiologic conditions18 and those under ischemic conditions19 has been also reported for the effect of brain-derived neurotrophic factor. These findings suggest that cAMP-CREB signaling has different modulatory actions on physiologic versus ischemia-induced neurogenesis in the hippocampal dentate gyrus.

In adult hippocampal neurogenesis, newborn cells show a progressive decrease in number within the first weeks, which stabilizes after 4 weeks.20 The time course of colocalization of pCREB and DCX in newborn immature neurons after ischemia was similar to that under normal conditions. We also found that ischemia simultaneously increased neurogenesis and neuronal elimination within a few weeks.20 In this study, we demonstrated that cAMP-CREB signaling was involved in the survival of newborn neurons after ischemia.

To activate the promoter of CREB target genes possessing the CRE sequence, phosphorylation of CREB at Ser 133 is crucial.5 Phosphorylation of CREB at this site is thought to recruit the coactivator CREB-binding protein (CBP), which functions by facilitating the interaction of CREB with the basal transcription machinery and by catalyzing the acetylation of chromatin of histone acetyltransferase activity. This activates transcription of genes containing the CRE sequence in their promoter.21 Therapeutic pharmacological approaches for targeting cAMP-CREB-CBP signaling include PDE4 inhibitors and histone deacetylase inhibitors.22 PDE hydrolyzes cAMP to AMP. Therefore, inhibition of PDE enhances cAMP-dependent CREB-CBP signaling. Among the PDE subfamily, PDE4 represents 70% to 80% of PDE activity in neuronal tissue.22 In neuron cultures, PDE4 tightly regulates cAMP formed by stimulation of N-methyl-d-aspartate receptors.23 The PDE inhibitor rolipram readily crosses the blood-brain barrier.24 Pretreatment with rolipram (3 mg/kg) decreases ischemic neuronal damage.25 In addition, administration of 1 mg/kg rolipram significantly enhanced hippocampal neurogenesis under normal conditions. Therefore, further studies will be needed to provide insight into the maximal effect of rolipram on neuronal protection and neurogenesis.

It was recently shown that rolipram treatment promotes axonal regeneration, attenuates glial scar formation, and enhances functional recovery after spinal cord injury.24 Rolipram also has been shown to improve synaptic and cognitive functions in the Alzheimer mouse model.26 Thus, activation of the cAMP-CREB pathway by rolipram may be an effective therapy for poststroke complications.

In this study, CRE-decoy treatment and rolipram might have indirectly influenced the cAMP-CREB cascade via expression of growth factors that are released from granule cells that surround pCREB-positive newborn neurons.6,7,17 Neurtrophins play crucial roles in adult neurogenesis after ischemia as well as under normal conditions. In particular, brain-derived neurotrophic factor and insulinlike growth fac-
tor promote adult neurogenesis,\textsuperscript{27,28} and both growth factors possess the CRE sequence in their promoters.

In summary, we have shown that pCREB expression is involved in the survival of newborn immature neurons in the adult hippocampus after ischemia. Pharmacological activation of cAMP-CREB signaling after ischemia by administration of the PDE inhibitor rolipram enhanced the survival of newborn neurons. These results indicate that pharmacological activation of cAMP-CREB signaling may provide a therapeutic approach for the treatment of stroke and poststroke complications.
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Disclosures
None.

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