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Erythropoietin Prevents Place Navigation Disability and Cortical Infarction in Rats with Permanent Occlusion of the Middle Cerebral Artery

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Erythropoietin (EPO) prevents the ischemia-induced delayed neuronal death in the hippocampal CA1 field in gerbils. EPO receptor (EPOR) is also expressed in the cerebral cortex but its function is not known. To examine whether EPO has a neuroprotective action in the cortex, EPO was infused into the cerebroventricles of stroke-prone spontaneously hypertensive rats with permanent occlusion of the left middle cerebral artery. Morris water maze test indicated that EPO infusion alleviated the ischemia-induced place navigation disability. The left (ischemic)-to-right (contralateral nonischemic) (L/R) ratio of cerebrocortical area in the EPO-infused ischemic group was larger than that in the vehicle-infused ischemic group. The occlusion caused secondary thalamic degeneration but infusion of EPO prevented the decrease in the L/R ratio of thalamic area and supported neuron survival in the ventroposterior thalamic nucleus. *In situ* hybridization indicated that EPOR mRNA was upregulated in the periphery (ischemic penumbra) of a cerebrocortical infarct after occlusion of the middle cerebral artery, suggesting that an increased number of EPOR in neurons facilitates the EPO signal transmission, thereby preventing the damaged area from enlarging.

Erythropoietin (EPO) produced by the kidney and the liver (in fetuses) is a glycoprotein that stimulates differentiation and proliferation of erythroid precursor cells, and hypoxic induction of EPO production increases the number of red blood cells, leading to better oxygen supply to tissues (1, 2). The action of EPO is mediated by binding to its specific receptor, a new family of cytokine receptors that have no tyrosine kinase domain (3).

Stimulation of red blood cell formation was considered to be the sole physiological function of EPO, but a novel neurotrophic function has also been proposed (4–6). EPO receptor (EPOR) is expressed in murine hippocampus and in primary cultured hippocampal neurons (5–7). Primary cultured astrocytes have been shown to produce EPO and low oxygen tension stimulates the production of EPO through an increase in its mRNA (8–10). Using a gerbil forebrain ischemia model, we have recently shown that intracerebroventricular EPO infusion prevents the delayed neuronal death in the hippocampal CA1 field and that the endogenous brain EPO plays an important role in the survival of hippocampal CA1 neurons under ischemia (11). EPOR is also expressed in cerebral cortex (5, 7) but its physiological significance remains unknown.

Although the delayed neuronal death in the hippocampal CA1 region of gerbils after transient forebrain ischemia is a well-established model (12–15), other ischemic models have been developed using rats (16–20). Among them, stroke-prone spontaneously hypertensive (SH-SP) rats with permanent occlusion of the middle cerebral artery (MCA) above the rhinal fissure and distal to the striate branches show a reproducible cortical infarct, place-navigation disability and secondary thalamic degeneration centered on the ventro-posterior nucleus (21–23). In addition, this rat model has an advantage over the gerbil model; it more closely mimics human patients with cerebrocortical infarction than does the gerbil ischemic model, and ischemia-induced behavioral abnormalities are easier to investigate in rats than in gerbils (19, 23). Thus SH-SP rats with permanent occlusion of MCA are pertinent to examine the effects of EPO on cerebrocortical ischemia. We now report the neuroprotective effect of

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EPO infused into the cerebral ventricle on place navigation disability, cortical infarction, and secondary thalamic degeneration of MCA-occluded SH-SP rats. We also show an occlusion-induced increase in EPOR mRNA by the use of *in situ* hybridization technique, which may partly account for the neuroprotective activity of EPO in the cerebral cortex.

MATERIALS AND METHODS

Erythropoietin. Recombinant human EPO (24) was dissolved in a vehicle consisting of 0.01 M phosphate-buffered saline (pH 7.5) and 0.1% bovine serum albumin. One unit of EPO corresponds approximately to 10 ng of EPO protein.

Animals. SH-SP rats at the age of 12–13 weeks, weighing 250-300 g, were housed in an air-conditioned room at a constant temperature (22 ± 1°C) with a 12:12 light-dark cycle, and food and water were given *ad libitum* throughout the experiment. The animals were handled once a week for cage cleaning. The following experiments were conducted in accordance with the Guide for Animal Experimentation at Ehime University School of Medicine. Tail systolic blood pressure in each conscious animal was measured before MCA occlusion and at 2 h , and 1, 2, 3, 4, 5, 6, and 7 days after MCA occlusion with the use of a rat tail manometer-tachometer system (KN-210, Natsume, Japan). The mean blood pressure of SH-SP rats before MCA occlusion was 206.8 ± 11.9 mmHg and it was not affected by MCA occlusion or EPO infusion.

Occlusion of the MCA and minipump implantation. SH-SP rats were anesthetized with 1.5% halothane in a 4:3 mixture of nitrous oxide and oxygen, while body temperature was kept at 37 ± 0.2°C. The left MCA was exposed by cutting the temporal muscle and then by making a hole in the temporal bone with a dental drill. Just after MCA occlusion, an osmotic minipump (Alza Corp., Palo Alto, CA) filled with either EPO or vehicle was implanted subcutaneously into the back of each animal and a needle from the minipump was placed in the left lateral ventricle. EPO in a dose of 0.2, 1, or 5 U/day was continuously infused through the minipump for 28 days (n = 8 per group). Sham-operated and MCA-occluded rats received vehicle infusion as controls (n = 8 per group).

Water maze test. Rats were subjected to repeated Morris water maze tests at the second and fourth week after MCA occlusion or sham operation. The experiments were carried out in a circular swimming pool (150 cm in diameter and 40 cm in depth), which was filled with water kept at 22°C and made opaque by the addition of milk. Four points on the rim of the pool were designated arbitrarily: north, south, east, and west, dividing the surface of the pool into 4 quadrants: north-east, north-west, south-east, and south-west. Local and distal cues in the room were fixed throughout the experiments. The movements of the animals were monitored and recorded with a video camera connected to a computer. The goal was a circular platform (12 cm in diameter) made of transparent plastic, and it was located 40 cm away from the wall in the north-east quadrant, and 2.5 cm beneath the water surface. Starting points were south and north, or west and east. Each trial ended when the rat had climbed onto the platform and stayed there for 10 s, or when it could not reach the platform within 90 s. Each test was carried out three times per day for 4 consecutive days. The escape latency, i.e., time until each rat reached the invisible submerged platform, was measured (21–23, 25). In cases where the rats could not escape onto the platform within 90 s, they were placed by hand onto the platform for 15 s and their escape latency was recorded as 90 s. The mean latency of finding the invisible platform was measured for individual animals on each day.

Morphological study. After the water-maze tests, the animals were anesthetized by an intraperitoneal injection of chloral hydrate (300 mg/kg), and the osmotic minipump was disconnected from the needle placed in the left lateral ventricle. Bromophenol blue was injected through the needle to confirm whether or not EPO and vehicle were successfully infused into the cerebral ventricles. Then the rats were perfused transcardially with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) and the brain was removed. The animals not showing dye diffusion into the cerebral ventricles were excluded from the experimental groups. The brains showing dye diffusion were embedded in paraffin. Three serial coronal sections 5 μm thick were cut at 1.2, 2.3, and 3.6 mm posterior to bregma and stained with 0.1% cresyl violet. The size of cortical infarct was assessed by measuring the area of the intact cerebral cortex in individual sections with a planimeter, and the ratio of the cerebrocortical area on the MCA-occluded side to that on the contralateral side was calculated in the three sections. This was termed L/R cerebrocortical ratio.

To assess secondary thalamic atrophy, the ratio of thalamic area on the MCA-occluded side to that on the contralateral side was calculated in the section 3.6 mm posterior to bregma. The thalamus at this level is known to constitute reciprocal fiber connections with the temporoparietal cortex, and thus it is likely to exhibit secondary degeneration after MCA occlusion that causes an infarct in the temporoparietal cortex. The degeneration of neurons in the ventroposterior (VP) thalamic nucleus was evaluated microscopically by measuring the size of each cell with a computerized image processing system (Nexus 6400 System; Kashiwagi, Tokyo, Japan). A square of 0.33 × 0.30 mm (510 × 478 pixels) was designated as a region of interest on the video image, and the size of each cell within the square was measured and expressed by its pixel size. Cells larger than 200 pixels were counted as viable neurons (21, 23, 26).

All experiments were done blindly with respect to the experimental group.

In situ hybridization. A rat EPOR cDNA fragment was obtained by using polymerase chain reactions and subcloned into pGEM-T vector (Promega, Madison, WI). This construct contained bases 45–804 of rat EPOR cDNA (starting codon is 1). The cDNA fragment was sequenced and confirmed to be identical with the corresponding gene (4). Antisense and sense probes were transcribed with the use of Sp6 and T7 polymerases in the presence of [^{35}S]UTP (NEN, Boston, MA). At 1, 8 and 24 h after MCA occlusion, the rats (n = 3 for each group) were decapitated under sodium pentobarbital anesthesia (50 mg/kg, i.p.). Normal rats (n = 3) were also decapitated under the same anesthetic condition. The brains were quickly removed and immediately frozen on powdered dry ice. Serial sections 20 μm thick were cut on a cryostat, thaw-mounted onto silane-coated slides, and stored at −80°C until use. The sections were air-dried and fixed for 15 min in 4% paraformaldehyde in 0.1 M phosphate buffer (PB), washed twice in PB for 5 min and treated with 10 mg/ml proteinase K in a solution containing 50 mM Tris–HCl and 5 mM EDTA for 5 min. After a rinse in PB for 5 min, the sections were fixed again in the same fixative for 5 min, then treated with 0.25% acetic anhydrate in 0.1 M triethanolamine for 10 min, washed in PB for 5 min, and dehydrated. Hybridization with the radioactive cRNA probes (5 × 10^6 dpm/ml) was performed at 55°C overnight in a solution containing 50% formamide, 20 mM Tris–HCl, 0.3 M NaCl, 1× Denhardt's solution, 10% dextran sulfate, 500 μg/ml yeast tRNA, and 200 μg/ml salmon sperm DNA. After hybridization, the sections were washed in 5× sodium chloride/sodium citrate (SSC), 1% mercaptoethanol (pH 7.0) at 55°C for 15 min and in high stringency wash solution containing 50% formamide, 2× SSC and 10% mercaptoethanol at 65°C for 30 min. They were washed three times in RNase buffer (10 mM Tris–HCl, 1 mM EDTA, 0.5 M NaCl) at 37°C (10 min for each) and treated with RNase A (1 mg/ml) in RNase buffer at 37° C for 30 min. Then, they were washed in RNase buffer for 10 min. After washing with the high stringency wash solution, the sections were washed in 2× SSC and in 0.1× SSC at room temperature for 10 min each, and dehydrated. The sections were coated with Kodak NTB-2 emulsion for liquid autoradiography (Kodak, Rochester, NY), exposed for 1–4 weeks and then developed. After washing in water and air-drying, they were counterstained with thionin, and coverslipped with Per-

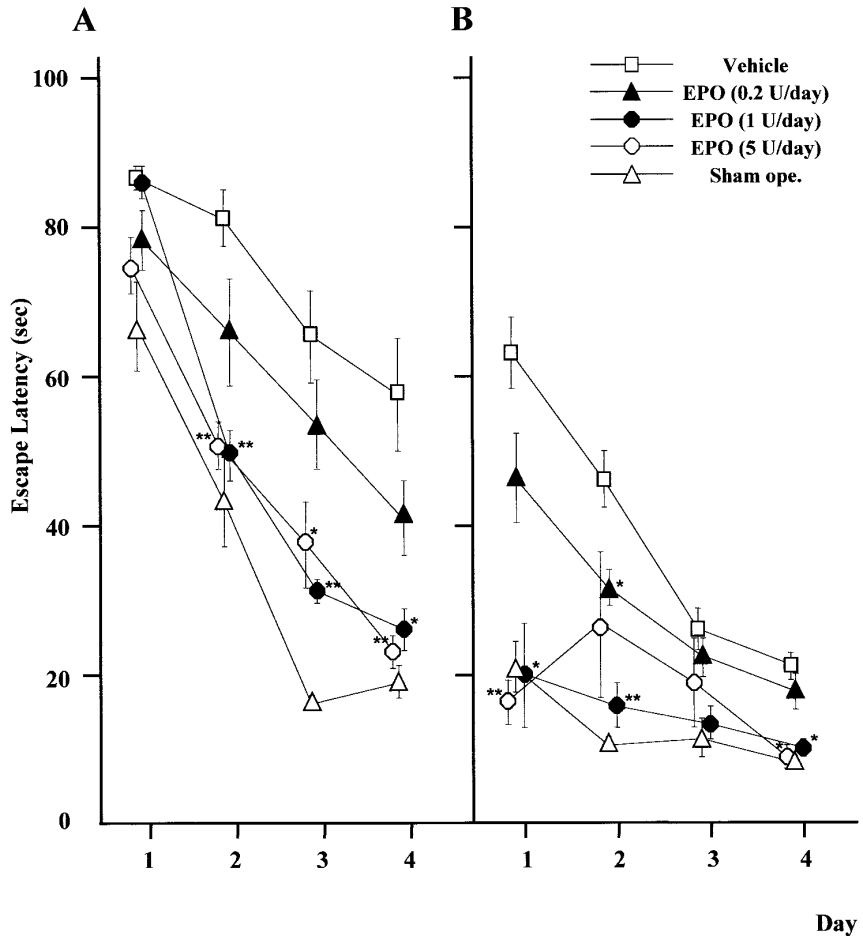

FIG. 1. Effect of EPO on place navigation ability of MCA-occluded rats. (A) Escape latency in Morris water-maze task at the second week after MCA occlusion. (B) Mean escape latency at the fourth week after MCA occlusion. MCA-occluded rats received intracerebro-ventricular infusion of the vehicle solution (□), 0.2 U/day EPO (▲), 1 U/day EPO (●) and 5 U/day EPO (○). (△) Sham-operated rats. All data are shown as mean ± SEM. *$p < 0.05$, **$p < 0.01$, significantly different from the vehicle-infused ischemic group (statistical significance tested by the analysis of variance, followed by post hoc test, Scheffe's *F*).

mount. Relative changes in mRNA expression were then quantified by determining, with the NIH image analysis system, the ratio of the optical density in the ischemic penumbra to that in the intact cerebral cortex.

RESULTS

To investigate the effect of infused EPO on place navigation ability of SH-SP rats with permanent occlusion of MCA, Morris water maze tests were conducted. Figures 1A and 1B show the results at the second and fourth week after MCA occlusion, respectively. At the second week after MCA occlusion, the mean escape latency of vehicle-infused ischemic rats was longer than that of sham-operated rats throughout the trial period (Fig. 1A). Treatment of MCA-occluded rats with 1 or 5 U/day of EPO significantly shortened the escape latency on the second to fourth trial days in comparison with the escape latency of vehicle-infused ischemic rats. The escape latency of ischemic rats treated with 0.2 U/day of EPO appeared to be shorter than that of the vehicle-infused ischemic rats but not significantly. At the fourth week after MCA occlusion there was also a significant difference in the escape latency between the vehicle-treated ischemic rats and the sham-operated rats (Fig. 1B). The ischemic rats treated with 1 U/day of EPO exhibited a significantly shorter escape latency than the vehicle-infused ischemic rats on the first, second, and fourth trial days, and those treated with 5 U/day of EPO on the first and fourth trial days. Infusion of 0.2 U/day of EPO prevented the ischemia-induced prolongation of escape latency but the difference was significant on the second trial day alone.

Among the experimental groups there were no significant differences in swimming speed (cm/s) (vehicle-infused rats, 31.4 ± 2.4; sham operated rats, 32.6 ± 1.8; EPO (0.2 U/day)-infused rats, 30.5 ± 3.2; EPO (1 U/day)-infused rats, 31.7 ± 2.5; EPO (5 U/day)-infused rats, 30.9 ± 1.8).

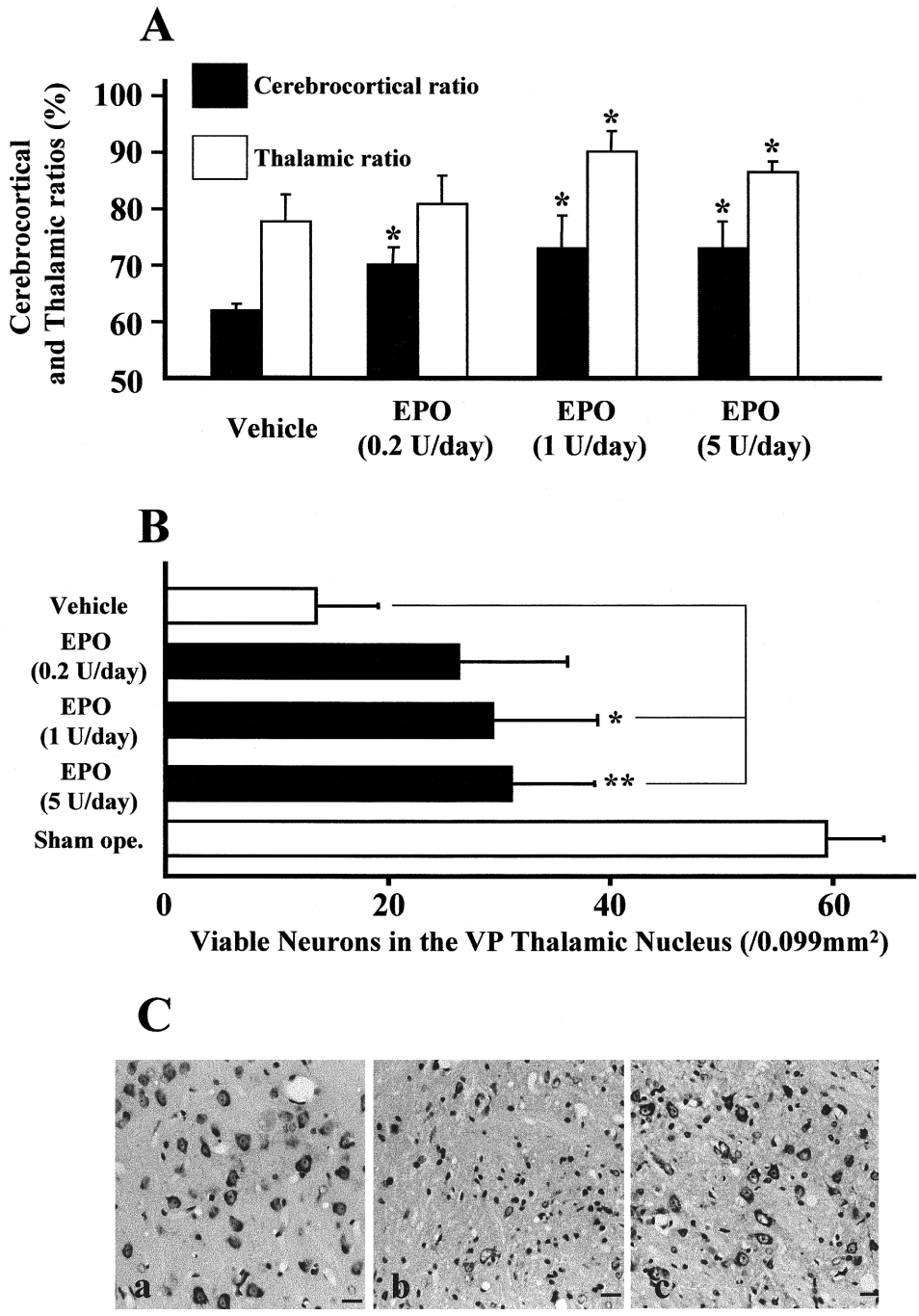

FIG. 2. Effect of EPO on cerebrocortical and thalamic areas of MCA-occluded rats. (A) The L/R ratios of cerebrocortical and thalamic areas in left MCA-occluded rats with or without EPO treatment for 28 days. (B) Number of viable neurons in the VP thalamic nucleus with or without EPO treatment. (C) Bright-field photomicrographs of the left ventro-posterior (VP) thalamic nucleus at the fourth week after MCA occlusion. a, sham-operated rat. b, MCA-occluded rat with vehicle infusion; c, MCA-occluded rat infused with 5 U/day of EPO. All data are shown as mean ± SD. *$p < 0.05$, **$p < 0.01$, significantly different from the vehicle-infused ischemic group (statistical significance tested by the two-tailed Mann–Whitney U test). Bars in C = 25 μm.

After the water maze tests at the fourth week after left MCA occlusion, the rats were processed for histological analysis of the brain. The left-to-right (L/R) ratio of cerebrocortical area in EPO-infused ischemic rats was larger than that of the vehicle-infused ischemic rats (Fig. 2A); 62.4 ± 1.1% for vehicle-infused rats, and 70.0 ± 3.6, 73.1 ± 7.3, and 73.2 ± 4.7% for the rats treated with EPO in the doses of 0.2, 1, and 5 U/day, respectively. Measurement of the L/R ratio of thalamic area indicated that EPO in the doses of 1 and

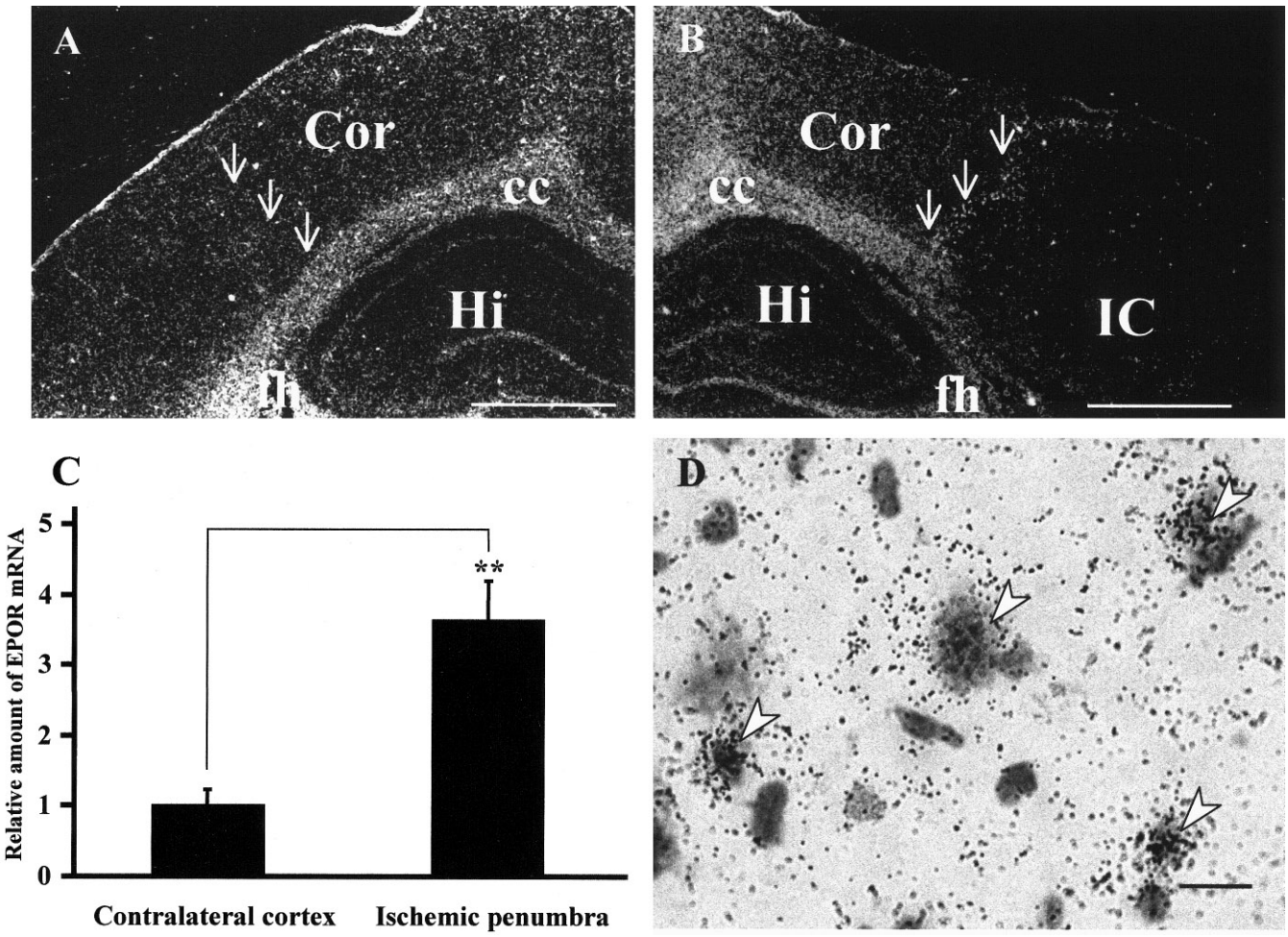

FIG. 3. Detection of EPOR mRNA by *in situ* hybridization histochemistry. (A and B) Dark-field photomicrographs showing EPOR mRNA in an intact (right) cerebral hemisphere (A), and in an MCA-occluded left cerebral hemisphere (B) at 24 h after MCA occlusion. Note an apparent upregulation of EPOR mRNA expression in the periphery (ischemic penumbra) of an infarct (arrows in B). cc, corpus callosum; Cor, cerebral cortex, fh, fimbria hippocampi; IC, ischemic core (infarct site); Hi, hippocampus. (C) Relative amount of EPOR mRNA in the ischemic penumbra and contralateral cerebral cortex at 24 h after MCA occlusion. The relative value in the ischemic penumbra was obtained by using the intensity of the corresponding area of the contralateral cerebral cortex as a standard, and is shown as the mean (±SD) of five independent analyses. $^{**}p < 0.01$, significantly different from the contralateral cortex (statistical significance tested by the tow-tailed Mann–Whitney *U* test). (D) A bright-field photomicrograph showing labeled neurons (open arrowheads). Bar = 1 mm (A, B), 25μm (D).

5 U/day significantly prevented the secondary thalamic degeneration in MCA-occluded rats; the ratio in the vehicle-infused ischemic rats was 77.4 ± 5.2%, while the ratios in rats treated with EPO in the doses of 0.2, 1, and 5 U/day were 81.8 ± 5.7, 96.7 ± 3.7, and 86.7 ± 2.2%, respectively.

Neuron counts using a computerized image processing system revealed that significant numbers of VP thalamic neurons were rescued from the secondary degeneration by treatment with 1 or 5 U/day of EPO (Fig. 2B). In comparison with sham-operated rats, Nissl staining of the vehicle-infused ischemic brain showed shrinkage and loss of thalamic neurons, and marked gliosis (Fig. 2C-a, b). Treatment with EPO apparently increased the number of VP thalamic neurons with a normal morphological appearance (Fig. 2C-c).

To investigate whether EPOR gene expression is influenced by MCA occlusion, we performed *in situ* hybridization histochemistry. In the nonischemic cerebral hemisphere contralateral to the MCA-occluded hemisphere, EPOR mRNA was intensely expressed in fiber tracts such as the corpus callosum and fimbria hippocampi (Fig. 3A). This pattern is similar to that in the sham-operated rats (not shown) and also to that of binding of radioiodinated EPO to brain sections (7) A moderate intensity of EPOR mRNA was also noted in the intact cerebral cortex. The sense probe did not show any hybridization signals, indicating that this probe specifically recognizes EPOR transcripts (data not shown). At 1 and 8 h after MCA occlusion, we did not detect any obvious changes in EPOR mRNA expression. At 24 h after MCA occlusion, an apparent

upregulation of EPOR mRNA expression was noted in the periphery (ischemic penumbra) of the infarct (Fig. 3B). Quantitative analysis indicated that the EPOR mRNA expression level in the ischemic penumbra was significantly higher than that in the contralateral cerebral cortex (Fig. 3C). At a high magnification, medium-sized neuronal cells in the ischemic penumbra abundantly expressed EPOR mRNA (Fig. 3D).

DISCUSSION

EPO has been demonstrated to protect hippocampal neurons from ischemia-induced death (11). EPOR has been reported to be expressed in the cerebral cortex (5, 7) but its function has not been determined. In this paper we showed that EPO infused into the cerebro-ventricles of MCA-occluded SH-SP rats alleviated the ischemia-induced place navigation disability and cortical infarction, suggesting that EPO in the cerebral cortex functions as a neurotrophic factor as it does in the hippocampus.

In developing the water maze task for the first time, Morris (25) reported that experimental animals with a hippocampal lesion exhibit place navigation disability. However, Hirakawa *et al.* (27) reported prolongation of passive and active avoidance responses in MCA-occluded rats, and Okada *et al.* (19) showed the occurrence of long-term spatial cognitive impairment (i.e., place navigation disability) in rats with MCA occlusion. We also showed that occlusion of the unilateral MCA above the rhinal fissure and distal to the striate branches in SH-SP rats causes place navigation disability (21–23). Thus, several brain regions including the hippocampus and the cerebral cortex may be responsible for cognitive impairment or place navigation ability. MCA occlusion in SH-SP rats caused the secondary degeneration of thalamic neurons and EPO infusion rescued these neurons. Thus the possibility can not be excluded that not only cortical neurons rescued by EPO but also thalamic neurons contribute to the amendment of place navigation disability in MCA-occluded rats. In support of this speculation, patients with cortical infarction after MCA occlusion develop dementia in association with retrograde thalamic degeneration (20). The thalamus may act as a relay center to convey sensory information necessary for the acquisition and/or maintenance of place navigation ability. If this is the case, the thalamus together with the cerebral cortex and hippocampus, is likely to control the place navigation behavior of rats. In agreement with this, a number of studies on behavioral and cognitive processes have demonstrated that multiple brain regions are involved in learning (19, 27).

EPO protects primary cultured hippocampal neurons from N-methyl-D-aspartate (NMDA) receptor-mediated glutamate toxicity (5), which is believed to be a major cause of neuron death by ischemia (28, 29). This neuroprotective action of EPO is exhibited by reducing the NO-mediated formation of free radicals or antagonizing their toxicity (11). It is likely that a similar mechanism operates in the action of EPO on cortical neurons, because EPO prevents NMDA receptor-mediated death of cultured cortical neurons (5).

Expression of EPOR was shown by immunostaining neurons with anti-EPOR antiserum (4–6) or by binding radioiodinated EPO to brain sections (7). To our knowledge, this is the first report of detection of EPOR mRNA in neurons by *in situ* hybridization. The spatial pattern of EPOR mRNA expression was similar to that obtained from binding of the radioactive EPO (7). Interestingly the expression was elevated in the ischemic penumbra at 24 h after MCA occlusion. This may help neurons acquire an increased resistance against ischemia through activation of the EPO signaling pathway. The mechanism underlying the MCA occlusion-induced expression of EPOR mRNA is not known.

In conclusion, the present study demonstrated that EPO ameliorates place-navigation disability, cortical infarction and thalamic degeneration in permanently MCA-occluded rats which exhibit a severer neuronal damage than experimental animals with transient forebrain ischemia (12, 30–32).

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