Cell Death Mechanism and Protective Effect of Erythropoietin after Focal Ischemia in the Whisker-Barrel Cortex of Neonatal Rats

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ABSTRACT

Cell death induced by the combined insult of hypoxia-ischemia in neonatal rodents has been extensively investigated. Ischemia-only-induced cell death, however, has been much less characterized. Based on the notion that 1) ischemic stroke is a relatively common disorder in human neonates, and 2) developing cells are more susceptible to apoptosis, the present study examined whether typical apoptosis was induced by cerebral ischemia in a new neonatal rat model. Erythropoietin (EPO; Epoetin) was tested for its protective effect against ischemia-induced cell death. Postnatal day 7 rats were subjected to permanent occlusion of the middle cerebral artery branch supplying the right whisker-barrel cortex. Terminal deoxynucleotidyl transferase biotin-dUTP nick end-labeled-positive cells in the ischemic region were detectable 4 h after ischemia and reached a peak level 16 h later. The cell death was preceded by caspase activation and cytochrome c release. Cell body shrinkage was evident among damaged cells. Agarose gel electrophoresis showed DNA damage with a smear pattern as well as DNA laddering. Electron microscopy demonstrated apoptotic features such as cell shrinkage, chromatin condensation, and fragmentation; meanwhile, necrotic alterations coexisted in the cytoplasm. EPO treatment increased signal transducers and activators of transcription-5 and Bcl-2 levels, markedly attenuated apoptotic cell death, and reduced ischemic infarct in the cortex. It is suggested that focal ischemia in the developing brain causes cell death with prominent apoptotic features coexisting with some characteristics of necrosis. This is consistent with the concept of hybrid death described previously in cultures and adult or developing brain. EPO may be explored as a potential therapy for neonatal ischemic stroke.

Ischemic stroke affects not only elderly but also young individuals. Recent studies have recognized that arterial ischemic stroke in infants and children is an important cause of morbidity and mortality and an emerging area for clinical and translational research (Lynch et al., 2002; Lee et al., 2005). The few days before and after birth are high-risk periods for stroke for both the mother and the infant. This is probably related to activation of coagulation mechanisms in this critical period. Perinatal arterial ischemic stroke is recognized in approximately one in 4000 full-term infants, which is higher than previously recognized (Nelson and Lynch, 2004). Specifically, neonatal stroke involving middle cerebral artery (MCA) occlusion may occur in term infants (Govaert et al., 2000; Ashwal and Pearce, 2001), and more than 80% of neonatal strokes involve the vascular territory supplied by the MCA (Ashwal and Pearce, 2001). Among ischemic stroke, focal cerebral ischemia may account for more cases of brain lesions in preterm and full-term neonates and may be more prevalent than global cerebral ischemia because of systemic asphyxia. Compared with investigations on adult ischemic stroke, much less attention has been paid to understanding the mechanism and treatment of perinatal, neonatal, and childhood strokes (Lynch et al., 2002).

Apoptosis has been identified as a typical mechanism of cell death in the developing brain. Features of apoptosis are prominent in the neonatal brain subjected to a hypoxia-
ischemia insult (Cheng et al., 1998; Nakajima et al., 2000). Apoptosis was also observed following transient focal ischemia in neonatal rats (Renolleau et al., 1998; Manabat et al., 2003). However, some reports suggest that the neuronal cell death induced by hypoxia-ischemia in neonates is not apoptosis (van Lookeren Campagne and Gill, 1996). A study on neonatal mouse hippocampus showed that even delayed cell death after hypoxia-ischemia does not have classic features of apoptosis (Sheldon et al., 2001). Whether typical apoptosis takes place after permanent cerebral ischemia in neonates remains elusive.

The ischemia-induced neonatal brain damage induced by transient or permanent cerebral ischemia has been studied in postnatal day 7 (P7) rat pups (Renolleau et al., 1998; Manabat et al., 2003; Wen et al., 2004). The MCA suture ligation used in these models successfully induces cell death in ischemic and penumbra regions and causes infarct in the ipsilateral hemisphere, including cortical and subcortical regions. Small strokes, on the other hand, often occur in clinical settings. To date, however, there has been no animal model in which small strokes are induced in the neonatal brain. Based on our previous “mini-stroke” model of whisker-barrel cortex in adult rats (Wei et al., 1995, 2001), we now report a new ischemic model of whisker-barrel cortex stroke in neonatal rats.

The hematopoietic factor erythropoietin (EPO; Epoetin) has been proposed as a potent neuroprotective drug in the treatment of ischemic stroke (Sakanaka et al., 1998; Bernaudin et al., 1999). Numerous studies have shown that EPO function is not limited to the hematopoietic system; EPO and functional EPO receptor (EPOR) have been identified in a variety of cells in rodents, primates, and humans (Digicaylioglu et al., 1995; Marti, 2004). EPO and EPOR are highly expressed in the developing brain, suggesting that they play important roles in neural development (Dame et al., 2000; Juul, 2002). A hypoxic-ischemic insult increased the expression of endogenous EPO and EPOR in the neonatal rat brain (Spandou et al., 2004), and permanent focal cerebral ischemia activated EPOR in the neonatal rat brain (Wen et al., 2004). EPO is neuroprotective against apoptosis induced by ischemic insults in vivo (Bernaudin et al., 1999; Digicaylioglu and Lipton, 2001; Wen et al., 2002) and against hypoxic-ischemic brain injury in neonatal rats (Kumral et al., 2003; Demers et al., 2005). It also improves functional and histological outcome in neonatal stroke in postnatal day 10 rats (Chang et al., 2005). Using the ischemic model of whisker-barrel cortex stroke, the present study investigated the cell death mechanism induced by a pure ischemic insult in neonatal rats and explored the potential neuroprotective effect of EPO against the ischemia-induced cell death. The effects of EPO treatment on the EPOR-JAK-2 downstream target signal transducers and activators of transcription-5 (STAT-5) and the antiapoptotic gene Bcl-2 were explored.

**Materials and Methods**

**Whisker-Barrel Cortex Ischemic Stroke in Neonatal Rats.** Wistar rats of P7 of both sexes were anesthetized by 3% isoflurane in a mixture of 70% N₂O and 30% O₂. Following induction of anesthesia, 1.5% isoflurane was maintained with mechanical ventilation. Body temperature was monitored and maintained at 36–37°C with a combination of overhead lights and a heating blanket. Surgical procedures to induce barrel cortex ischemia in adult rats have been reported previously (Wei et al., 1995, 2001), barrel cortex ischemia in this investigation was induced similarly with modifications for neonates. Animals were placed in a noninvasive head-holder, and a 2.5- to 3.0-mm-diameter craniectomy was performed through the right parietal skull, and the transparent dura was left intact over the whisker-barrel cortex. Under a dissecting microscope, sterile no. 11 silk sutures were passed through the dura to ligate a proximal branch of MCA. To ensure sufficient reduction in the blood supply, the common carotid artery was additionally occluded for 10 min. Most likely because of the less developed collateral circulation in the neonate, this ligation method was sufficient to induce a selective damage in the barrel cortex (Fig. 1). In sham controls, ligatures were placed through the dura and under arterial branches and common carotid artery but not tied. After surgery and waking up, the pups were returned to their mothers for recovery. Recombinant EPO (Amgen Biologicals, Thousand Oaks, CA) was i.p. injected according to the experimental plan.

**Ischemic Infarct Volume Assay.** Postsurgery rats were subsequently sacrificed at various times after ischemia. Animals were killed with an overdose of pentobarbital (100 mg/kg) followed by intracardiac perfusion with 200 ml of 0.9% NaCl. Brains were then sliced into 2-mm coronal sections using a rat brain matrix (Harvard Bioscience, South Natick, MA). The cortical infarct volume was morphometrically measured after triphenyl tetrazolium chloride (TTC) staining. The brain slices were incubated in phosphate-buffered saline (pH 7.4) containing 2% TTC at 37°C for 20 min and then stored in 10% neutral-buffered formalin. A cross-sectional area of the TTC-unstained region for each brain slice was determined using an image analyzer (MCID Imaging System; Imaging Research Inc., St. Catharines, ON, Canada) and the indirect method (subtraction of residual right hemisphere cortical volume from the cortical volume of
the intact left hemisphere). Hemispheric cortical volume was calculated by summation of infarct volumes measured in component brain slices. Infarct assessments were performed under blind conditions.

**H&E Analysis.** After sacrifice, animals were perfused with 4% paraformaldehyde and brain 8-μm sections were prepared for H&E staining following standard histochemical procedures (Luna, 1968).

**Terminal Deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling.** Animals under anesthesia underwent perfusion fixation with 200 ml of normal saline followed by 10% formalin at different hours after ischemia. Paraffin-embedded brain sections of 10 μm were deparaffinized in two changes of xylene for 5 min each and then washed sequentially in 100, 95, and 70% ethanol. Nuclei of tissue sections were stripped of proteins by incubation with 20 μg/ml proteinase K (Sigma-Aldrich, St. Louis, MO) for 5 min. Slices were washed in distilled water. The new 3'-OH DNA ends generated by DNA fragmentation typically localized in nuclei were stained using the ApopTag in situ apoptosis detection kit (S7110-kit; Oncor Inc., Gaithersburg, MD). For double staining with Hoechst, brain sections were then incubated with 10 μM Hoechst 33285 for 2 min.

**Caspase-3 Activation of Immunohistochemical Staining.** Activated caspase-3 was visualized by CM1 antibody or caspase-3 immunoassay/activity kit (Calbiochem, San Diego, CA). The rabbit polyclonal antiserum CM1 recognizes the p18 subunit of cleaved caspase-3 and has been used as a specific detector for caspase-3 activation. Frozen 10-μm-thick brain sections were fixed and then incubated with 2% Triton X-100 for 30 min followed by PBS wash. Brain sections were processed for peroxidase immunohistochemistry using the CM1 antibody (1:20,000) with the VECTASTAIN Elite ABC kit (Vector Laboratories, Burlingame, CA) or incubated with anticytochrome-c antibody (1:100; 1 h), rinsed in PBS three times. For NeuN double labeling, additional incubation with the NeuN antibody (1:200; 2 h) was performed. After washing, sections were counterstained with bisbenzimide (5 min and rinse) and coverslipped in VECTASHIELD. Cell staining was visualized using a Nikon fluorescence microscope (Nikon TE-2000; Nikon, Melville, NY) and photographed.

Caspase catalytic activity was also measured as described previously (Han and Holtzman, 2000). Briefly, tissue homogenates were lysed in 80 μl of buffer A (10 mM HEPES, 42 mM KCl, 5 mM MgCl₂, 1 mM dithiothreitol, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, and 1 μg/ml leupeptin, pH 7.4). Lysate (10 μl) was combined in a 96-well plate with 90 μl of buffer B (10 mM HEPES, 42 mM KCl, 5 mM MgCl₂, 1 mM dithiothreitol, 1% Triton X-100, and 10% sucrose, pH 7.4) containing fluorometric substrate (30 μM) and incubated for 45 min at room temperature in the dark. Formation of fluorogenic product was determined in a CytoFluor fluorometric plate reader by measuring emission at 480 nm with 360-nm excitation. Caspase-3-like activity was correlated with cleavage of N-acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin (DEVD-AMC).

**Cytochrome c Immunohistochemistry.** Anesthetized animals were perfused with 10 U/ml heparin and subsequently with 4% formaldehyde in 0.1 M PBS, pH 7.4. The brains were removed, postfixed for 12 h, sectioned at 25 μm on a vibratome, and processed for immunohistochemistry. The sections were incubated with a blocking solution and reacted with rabbit anti-cytochrome c polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at a dilution of 1:200. Primary antibody binding was detected by incubating the sections with the VECTASTAIN Elite ABC kit (Vector Laboratories). As a negative control, sections were incubated without primary antibodies.

**Determination of DNA Fragmentation (Laddering).** Homogenates of the ipsilateral cortex (ischemic and penumbra regions) were obtained from the sham-operated and ischemic stroke animals, resuspended in lysis buffer (10 mM Tris-HCl, 100 mM EDTA, and 0.5% SDS, pH 8.0) for 5 min at room temperature, and then treated with proteinase K (300 μg/ml) for 2 h at 50°C. DNA was precipitated overnight at 4°C by adding NaCl to a final concentration of 1 M. The lysate was centrifuged at 13,000 rpm for 1 h at 4°C followed by extraction of DNA with phenol/chloroform/isoamyl alcohol (25:24:1). The total DNA contained in the aqueous phase was precipitated with isopropanol. The DNA pellet was washed twice with 70% ethanol and resuspended in TE buffer containing RNase at 0.3 mg/ml. Aliquots (10–15 μg of DNA) were analyzed on a 1.5% agarose gel and run at 75 V for 3 h. Following electrophoresis and staining with ethidium bromide, the gel was visualized under ultraviolet light and photographed.

**Cell Surface Area and Cell Death Assessments.** Cell surface area was assessed using the imaging analysis software ANALYSIS (Soft Imaging System Corp., Lakewood, CO) on 10 randomly chosen nonoverlapping fields of three cortical areas of the ipsilateral and contralateral hemispheres under 40× magnification.

Cell death in the ischemic areas of coronal brain sections was evaluated by counting TUNEL and activated caspase-3-positive cells and their ratios to the total cells counterstained with Hoechst 33285 or propidium iodide. Ten nonoverlapping microscopic fields of three cortical areas of the ipsilateral hemisphere were counted in 1.25-mm² areas in the ischemic region under 40× magnification. A stereological count of total cells and double-labeled cells was conducted on every 10th section so cell counting was performed on slices 90 μm apart to avoid repeated counting of the same cells. Cell counts were confirmed by additional counting on confocal images. Average numbers and ratios of a cell type to total cells per field were calculated.

Cell surface assay and cell counting were performed under blind condition.

**Western Blotting of STAT-5 and Bcl-2.** Tissue samples were taken from the ipsilateral cortex with and without EPO treatment. Proteins were extracted using the NE-PER Nuclear and Cytoplasmic Extraction kit (Pierce Chemical, Rockford, IL). Protein concentration of each sample was determined using the bicinchoninic acid assay (Sigma-Aldrich). Proteins from each sample (50 μg) were separated by SDS-polyacrylamide gel electrophoresis in a Hoefer Mini-Gel system (Amersham Biosciences, Inc., Piscataway, NJ) and transferred in the Hoefer transfer tank (Amersham Biosciences, Inc.) to a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA). Membranes were blocked in 7% evaporated milk diluted in Tris-buffered saline containing 0.1% Tween 20 (TBS-T) at room temperature for at least 2 h and then incubated with polyclonal rabbit anti-phospho-STAT-5 (Santa Cruz Biotechnology, Inc.) or polyclonal rabbit anti-Bcl-2 antibody (Oncogene Science, Cambridge, MA). A mouse β-actin antibody (Sigma-Aldrich) was used for protein loading control. After primary antibody incubation, membranes were washed with TBS-T and incubated with alkaline phosphatase-conjugated anti-rabbit IgG (Promega, Madison, WI) antibody for 2 h at room temperature. Finally, membranes were washed with TBS-T followed by three washes with Tris-buffered saline, and signal was detected by the addition of BCIP/NBT solution (Sigma-Aldrich).

**Electron Microscopy.** For ultrastructural examination, sacrificed rats were perfused intra-aortically with glutaraldehyde 2% in 0.1 M phosphate buffer. After perfusion, the brains were removed and postfixed for 1 day in the same solution. Ultrathin sections through dentate gyrus and cerebral cortex were cut on a Reichert Ultracut ultramicrotome (Mager Scientific Inc., Dexter, MI), mounted on 150-mesh copper grids, and poststained in uranyl acetate and Reynolds’s lead citrate. Sections were photographed using a transmission electronic microscope (Zeiss 902; Carl Zeiss Inc., Thornwood, NY) (Weil et al., 2004).

**Statistics.** Student’s two-tailed t test was used for comparison of two experimental groups. In the event that there was a doubt that samples might not come from normally distributed populations, rank sum tests were performed using SigmaStat (SYSTAT Software, Inc., Point Richmond, CA). Multiple comparisons were analyzed using one-way analysis of variance followed by a post hoc Tukey test for multiple pairwise examinations. Changes were identified as significant if P was less than 0.05. Mean values were reported together with the standard deviation.
Results

Focal Ischemic Stroke in the Neonatal Barrel Cortex. In the P7 newborn rat, permanent occlusion of the MCA branch supplying the whisker-barrel cortex caused a visible ipsilateral cortical infarct 12 h after the insult (Fig. 1). H&E staining showed significant cell damage in the ischemic region 6 h after the ischemic insult; injured cells looked dark and showed shrunken cell bodies with condensed nuclei (Fig. 1). In the ischemic region, the apoptotic feature of cell shrinkage was evident; the cell surface area became much smaller than that in the contralateral side of the brain (Fig. 2), which was a sign of apoptotic injury.

Cell death was further identified using TUNEL staining. Noticeable TUNEL-positive cells were seen in the ischemic region 4 h after initiation of ischemia and reached a peak level 24 h after the insult (Fig. 3, A and B). The TUNEL positive cells had shrunken cell bodies and condensed nuclei. Specifically, nuclear fragmentation was seen in many TUNEL-positive cells (type II TUNEL-positive cells) (Wei et al., 2004), which indicated an apoptotic nature or an apoptotic component of the injury (Fig. 3A). Conversely, only a few TUNEL-positive cells were detected in the normal or contralateral side of the brain (data not shown).

Ischemia-Induced Hybrid Cell Death of Apoptotic and Necrotic Changes in Neonatal Brain. To confirm the apoptotic nature of the cell death, caspase-3 activation in the ischemic brain was visualized with the anti-CM-1 antibody or the anti-caspase-3 polyclonal antibody, which recognizes the activated form of caspase-3 (Fig. 4, A and B). To quantify the catalytic activity of activated caspase-3, we also measured the cleavage of DEVD-AMC. Evidence of caspase-3 activation was seen in the ipsilateral but not the contralateral cortex. Activation reached a plateau level about 8 h after ischemia and persisted for about 1 day before gradually declining toward the normal level (Fig. 4C). The ischemia-induced cell death was also characterized by cytochrome c release. Using immunohistochemical staining, cytochrome c in the cytoplasm was detected 6 to 12 h after ischemia (Fig. 5).

Apoptosis is characterized by DNA laddering of ~180-base fragments. DNA damage of TUNEL-positive cells after ischemia in the neonatal cortex. A, TUNEL-positive cells (green) in the ischemic region 16 h after ischemia. Some cells show characteristics of DNA fragmentation (arrows), which are typical for the type II TUNEL-positive cells of apoptotic damage. Blue color is Hoechst staining of all cells. B, cell death in ischemic cortex was detected by TUNEL staining 12 h after ischemia. Cell death increased about 4 h after ischemia and reached a peak level around 16 to 48 h after ischemia. n = 8 to 10 animals for each time point (three brain sections per animal).

Fig. 2. Focal ischemic-induced cell shrinkage in the ischemic region. Cell surface area was measured 16 h after ischemic insult using the ANALYSIS imaging software. A marked decrease in the cell surface area, likely represented by a cell volume decrease, was seen in the ischemic cortex compared with that in the same region of the contralateral side. Cell assay was performed under 40× magnification in four nonoverlapping fields. n = 10 animals (three brain sections per animal); *, P < 0.05 compared with no ischemia control by Student’s t test and by rank sum tests.

Fig. 3. DNA damage of TUNEL-positive cells after ischemia in the neonatal cortex. A, TUNEL-positive cells (green) in the ischemic region 16 h after ischemia. Some cells show characteristics of DNA fragmentation (arrows), which are typical for the type II TUNEL-positive cells of apoptotic damage. Blue color is Hoechst staining of all cells. B, cell death in ischemic cortex was detected by TUNEL staining 12 h to 14 days after ischemia. Cell death increased about 4 h after ischemia and reached a peak level around 16 to 48 h after ischemia. n = 8 to 10 animals for each time point (three brain sections per animal).

Fig. 4. Caspase activation after ischemic insult. A, caspase-3 activation was detected using the CM-1 antibody. Noticeable CM-1-positive cells (dark brown) were seen in the ischemic region 12 h after ischemia. B, an enlarged image from the ischemic area in A. C, time course of caspase-3 activity measured by DEVD cleavage. Significant caspase activation started from 4 h after ischemia and reached a peak level 8 to 24 h after ischemia. The gradual reduction in caspase activation was likely a combination of reduced enzyme activity and cell death. n = 8 to 10 animals for each time point (three brain sections per animals). *, P < 0.05 compared with sham control.
pair breaks detectible on an agarose gel by electrophoresis. During the first few hours after ischemia, DNA isolated from the ipsilateral cortex in the region of the ischemia showed predominantly a smear on agarose gels, suggesting a necrotic injury. From 8 h postischemia, DNA laddering became visible in the ipsilateral brain. Apoptotic-like DNA fragmentation was more evident 16 h after ischemia (Fig. 6).

To confirm morphological features of cell death and to verify whether necrotic and apoptotic alterations evolved in parallel with each other or in sequence, electron microscopy was performed 8, 16, and 24 h after the onset of ischemic insult. Damaged neurons showed marked cytoplasm shrinkage, nuclear/chromatin condensation, and a large amount of fragmentation, consistent with apoptotic morphology (Fig. 7). In later stages, apoptotic bodies of intact membrane containing condensed dark chromatin masses formed in many cells; some apoptotic bodies even fell off the cells (Fig. 7). On the other hand, it was also evident that, at all time points examined, most cells showed vacuolated cytoplasm coexisting with condensed nuclei and/or apoptotic body formation. The deteriorating cytosol and disrupted membranes indicated a necrotic component throughout the time period after ischemia. Thus, concurrent morphological features of both apoptosis and necrosis, or hybrid cell death, took place after the cerebral ischemia (Fig. 7).

Neuroprotection of EPO against Ischemia-Induced Cell Death and Infarct Formation in the Neonatal Brain. Systemic administration of recombinant EPO (10,000 U/kg i.p.) was given to animals 5 to 10 min before MCA occlusion and repeated once a day after ischemia. No gross adverse effects of EPO were observed. The EPO treatment significantly reduced caspase-3 activation (Fig. 8, B and C) and the percentage of TUNEL-positive cells (Fig. 8, B and D). Consistently, EPO showed a remarkable protective action of reducing ischemic infarct volume (Fig. 8, A, E, and F). Suggesting a lasting EPO protection, the brain weight of EPO-treated rats measured 5 weeks after ischemia was significantly greater than that of time-matched saline control rats (1.67 ± 0.02 versus 1.54 ± 0.02 g; n = 7 and 6, respectively;
P < 0.05; EPO 10,000 U/kg i.p. before ischemia and once a day for 10 days after ischemia).

**EPO Induced Increases of STAT-5 and Bcl-2 Expression in the Ischemic Brain.** To understand the possible mechanism mediating the EPO neuroprotection, we examined the level of phosphorylated STAT-5, a downstream signal of the EPO-JAK-2 pathway (Chong et al., 2002). Examined at 12 and 24 h after ischemia, animals that received EPO injection (10,000 U/kg i.p. before ischemia) showed increased level of STAT-5 in the ipsilateral cortex compared with that of ischemia-only animals (Fig. 9A). EPO treatment also enhanced expression of the antiapoptotic gene Bcl-2 in the ipsilateral cortex (Fig. 9B).

**Discussion**

Although extensive investigations have studied neonatal brain damage induced by combined insults of hypoxia plus ischemia, neonatal stroke induced by ischemia alone has been much less studied. The nature of neonatal cell death after an ischemia insult remains obscure. The present investigation examined the neuronal death solely induced by permanent ligation of the MCA branch supplying to the cerebral sensorimotor cortex of neonatal rats. Marked apoptotic alterations are identified as a primary feature of neuronal cell death in this ischemia model. Apoptosis was demonstrated as shrunken cells that were positive for TUNEL and caspase-3 staining, cytochrome c release, DNA laddering, and characteristic ultrastructural changes. Meanwhile, a noticeable necrotic component was revealed by DNA smearing, vacuolated cytoplasm, and collapsed membranes at subcellular and ultrastructural levels. These observations correspond with a previous study of hypoxia-ischemia-induced hybrid cell death in the newborn rat (Nakajima et al., 2000). EPO, as shown in various other animal model studies, exhibited significant neuroprotective effects. EPO treatment reduced infarct volume 3 days after ischemia (Fig. 9E). EPO treatment markedly attenuated the ischemia-induced infarct volume 3 days after ischemia (Fig. 9F).
neuroprotection against ischemic infarct formation and cell death in the neonatal brain. The result is consistent with a very recent report showing that, after focal cerebral ischemia, EPO activates several pathways and attenuates brain injury in P7 rats (Sola et al., 2005).

Neonatal stroke animal models are essential for understanding the mechanism of cell death and exploring potential therapeutic treatments in neonatal stroke. The most commonly used rodent model of neonatal stroke is the combined insult of hypoxia-ischemia in rats at postnatal day 7. This model is performed by ligation of unilateral carotid artery and exposure to systemic hypoxia for 2 to 3 h, which results in a reproducible unilateral brain damage ipsilateral to the ligated artery. Many pathophysiological mechanisms related to hypoxia-ischemia have been studied using these models (Yager and Thornhill, 1997; Cheng et al., 1998; Ashwal and Pearce, 2001). The P7 rats are used based on the notion that the developmental stage of these pups corresponds to that of a near term human (McIlwain and Bachelard, 1971). The hypoxia-ischemia model, however, requires the application of systemic hypoxia that may not imitate the pathophysiological condition of pure focal ischemia. Ligation of extracranial vessels with superimposed hypoxia in this model is also different from obstruction of cerebral blood flow of focal stroke in human neonates. To understand the ischemia-induced injury in the developing brain, ischemic stroke neonatal animal models have been used in some previous investigations (Renolleau et al., 1998; Manabat et al., 2003; Wen et al., 2004). The neonatal model in the present study was developed based on our previous barrel stroke model in adult rats (Wei et al., 1995, 2001). The MCA branch occlusion used in the present investigation provides an ischemic stroke model of neonates that mimics small strokes often observed in clinical cases.

The developing brain is different from the mature brain in a number of structural and functional characteristics (Johnston et al., 2002; Herlenius and Lagercrantz, 2004). During development, brain susceptibility to hypoxia and ischemia varies greatly (Muramatsu et al., 1997; Yager and Thornhill, 1997). Compared with the adult brain, the neonatal brain shows higher tolerance to hypoxic and ischemic insults (Singer, 1999; Johnston et al., 2002); on the other hand, neurons in the neonatal brain are more susceptible to die from apoptosis (Dikranian et al., 2001; Johnston et al., 2002). The development of typical necrotic and apoptotic cell death by distinct insults has been explicitly demonstrated in vitro studies. The nature of ischemic cell death in vivo, however, has not been well defined. Increasing evidence indicates that, except for severe attacks such as cell death in the ischemic core region of adult animals, a mixed form of necrosis and apoptosis emerges as a common pathological occurrence (Eichenbaum et al., 2002; Liu et al., 2004; Wei et al., 2004). Our previous studies showed concurrent apoptotic and necrotic alterations in the same cells after different insults such as energy deficiency, reactive oxygen induction in vitro, and focal ischemia in adult animals (Xiao et al., 2002; Wei et al., 2004). During normal development, classic apoptosis occurs in the neonatal brain. In the setting of neonatal brain injury, cells can die with many features of apoptosis, although there are morphological differences between natural cell death during development versus in the setting of injury. The present study largely supports the idea that cell death with apoptotic features is a primary form of neuronal injury induced by focal ischemia in the neonatal brain. However, even in the neonatal brain, a necrotic component coexists in the same cells as those with apoptotic features. We agree with the notion that detection of an apoptotic process such as caspase activation and/or DNA laddering does not conclude typical apoptotic cell death. On the other hand, some coexisting necrotic alterations should not renounce apoptosis in the death mechanism. The characteristics of hybrid cell death imply that therapies that target both apoptosis and necrosis are necessary to protect neurons against ischemic insults in adults as well as in neonates.

As a downstream signal of hypoxia-inducible factor-1α, EPO increases after ischemic or hypoxic insults (Bernaudin et al., 1999; Dicicciayloglu and Lipton, 2001; Marti, 2004). Recent studies in neonatal rats showed a significant increase in EPO receptor protein in the ischemic areas 6 to 12 h after permanent focal cerebral ischemia (Wen et al., 2004). Endogenous EPO has also been shown to increase in the ipsilateral cortex 1 to 7 days after hypoxia-ischemia (Spandou et al., 2004; Sun et al., 2004). EPO showed marked neuroprotection against brain injury, especially apoptosis, after neonatal hypoxia-ischemia, partially mediated by the activation of HSP27 (Kumral et al., 2003; Sun et al., 2004). EPO increases the expression of the antiapoptotic gene Bcl-xL and decreases the expression of the proapoptotic gene Bak in PC12 cells (Renzi et al., 2002). EPO may regulate the balance of the Bcl/Bax ratio toward a net antiapoptotic effect in cultured microglia and neurons (Vairano et al., 2002; Wen et al., 2002). Therefore, EPO inhibits caspase activities linked to cytochrome c release (Chong et al., 2003).

Although the JAK-2-STAT-5 pathway is well established as a downstream signal of EPO receptor activation, its role in EPO-induced neuroprotection in the ischemic brain remains poorly defined. Our study provides new evidence that EPO treatment in vivo activates STAT-5 signaling. We showed that EPO enhanced the phosphorylated STAT-5, which could not only in turn increase antiapoptotic gene Bcl-xL (Wen et al., 2002; Sola et al., 2005) but also increase another major antiapoptotic gene Bcl-2. The EPO-increased antiapoptotic genes and its inhibitory effect on caspase activation afford a prominent antiapoptotic action. Because EPO has been shown to be protective against both apoptosis and necrosis (Joyeux-Faure et al., 2005), the present study strongly supports the possibility that EPO and its analogs may be explored as therapeutic drugs for the treatment of neonatal stroke.

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