The Effect of Recombinant Human EPO on Neurovasculature Repair after Focal Ischemic Stroke in Neonatal Rats

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List of non-standard abbreviations:

rhEPO, recombinant human erythropoietin
EC, endothelial cell
BrdU, 5-bromo-2’-deoxyuridine
GLUT-1, glucose transporter-1
NeuN, neuronal nuclear protein
EPOR, erythropoietin receptor
HIF-1α, hypoxia-inducible factor-1α
bFGF, basic fibroblast growth factor
Ang, angiopoietin
VEGF, vascular endothelial growth factor
BCIP/NBT, 5-bromo,4-chloro,3-indolylphosphate nitroblue tetrazolium

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Abstract

Cerebral ischemia disrupts the neurovascular unit, involving death of neuronal, glial and endothelial cells (ECs) in the core and penumbra regions. While the neuroprotective effect of recombinant human erythropoietin (rhEPO) has been widely investigated, its effects on ECs remain elusive. We now report the effects of rhEPO treatment on EC death and neurovasculature repair following a focal ischemic stroke in postnatal day 7 neonatal rats. rhEPO (5,000 U/kg i.p.) was administered 60 min after ischemia and for the next 3 days. Western blot analysis revealed increased expression of neurovascular remodeling proteins including Tie-1, angiopoietin-2, and bFGF in rhEPO-treated pups. rhEPO treatment significantly reduced EC death in the ischemic penumbra region 12–72 hrs after ischemia examined by immunostaining of TUNEL and EC marker glucose transporter-1 (GLUT-1). Treatment with rhEPO increased proliferation of ECs and neuronal cells, revealed by co-staining of 5-bromo-2’-deoxyuridine (BrdU) with GLUT-1 or with the neuronal marker NeuN 7-21 days after stroke. Specifically, rhEPO increased number of NeuN-positive cells in close proximity to proliferating microvessels. These results suggest for the first time that, in addition to its protection on neural cells, EPO protects ECs and promotes the neurovascular unit repair, which may contribute to its therapeutic benefits after neonatal ischemic stroke.
Introduction

Neonatal stroke has increasingly been recognized as a significant cause of mortality and long-term neurological deficits in newborns; specifically, arterial ischemic stroke around the time of birth affects about one in 4,000 full-term infants (Nelson and Lynch, 2004; Ozduman et al., 2004). The developing brain differs from the adult brain both structurally and functionally (Johnston et al., 2001; Wei et al., 2006) and in its response to hypoxia and ischemia (Derugin et al., 2000). Newborn cells in parts of the brain may differ morphologically and functionally from cells produced in the adult brain (Lemasson et al., 2005). Compared to the adult brain, the developing brain exhibits higher plasticity and is relatively resistant to excitotoxicity (Johnston et al., 2001; Blomgren et al., 2003).

The type of cell death that occurs following a hypoxic and/or ischemic event varies substantially between the adult and the neonatal brain. Comparisons of adult and immature stroke models suggest apoptosis and an apoptosis/necrosis continuum may be more prevalent in the neonatal brain, and apoptotic cell death is most likely responsible for delayed cell death (Johnston et al., 2001; Northington, 2006; Wei et al., 2006). The developing brain and immature cells are more susceptible to apoptotic death, which has been defined as an important pathological process in a variety of neonatal models including hypoxia-ischemia (Hill et al., 1995; Nakajima et al., 2000) and transient or permanent focal cerebral ischemia (Wen et al., 2004; Wei et al., 2006). The functional and developmental differences described between the neonatal and adult brain indicates that extrapolating adult data to the neonate is not recommended.
Angiogenesis, the growth of new blood vessels from an existing vasculature, may contribute to neuroprotection and functional recovery after stroke. Recent studies suggest that vascular endothelial cells (ECs), neurons, and astrocytes that are in physical proximity to the endothelium form a functional unit that serves to maintain cerebral homeostasis. Physiological interactions between all these components of the neurovascular unit control cerebral microcirculation; the structural and functional integrity of the unit is critical for normal brain function as well as tissue repair after brain damage (Blomgren et al., 2003; Curin et al., 2006). Therefore, in addition to neural cells, protection and regeneration of ECs and ultimately the neurovascular unit has emerged as a potential approach in the treatment of ischemic stroke. The idea of the neurovascular unit has been further supported by studies showing that proteins originally thought to be only involved in angiogenesis, such as the Tie/Ang system, may be involved in modulating neurogenesis as well (Ohab et al., 2006; Zhang et al., 2007).

Erythropoietin (EPO) is a hematopoietic glycoprotein that induces homodimerization of two molecules of the EPO receptor (EPOR) on the cell surface (Farrell and Lee, 2004), initiating the Janus kinase/signal transducers and activators of transcription signal transduction cascade (Wei et al., 2006; Zhang et al., 2007). Despite the differences between the adult and the developing brain, studies in both adult and neonatal humans and rodent stroke models have shown the safety and efficacy of rhEPO treatment (Ehrenreich et al., 2002; Gumy-Pause et al., 2005; McPherson et al., 2007). In our previous investigations, we showed that recombinant human erythropoietin (rhEPO) was protective against ischemia-induced neuronal cell death in neonatal rats (Wei et al., 2006) and stimulated angiogenesis that led to restoration of local blood flow after
ischemia in adult rodents (Li et al., 2007). rhEPO has also been implicated in vascular protection (Chong et al., 2002; Li et al., 2007). In addition, EPO administration significantly increased neurogenesis in the dentate gyrus following brain injury (Grote et al., 2005). The upregulation of EPO and EPOR in blood vessels, neurons, and astrocytes of adult human brains infers their role in endogenous protective mechanisms (Sola et al., 2005).

Ischemia increases expression of hypoxia-inducible factor-1α (HIF-1α) and results in the transcriptional activation of VEGF (Shweiki et al., 1992) and basic fibroblast growth factor (bFGF) (Folkman and Klagsbrun, 1987). Angiopoietins (Ang-1, Ang-2) and their receptors (Tie-1, Tie-2) are angiogenic factors involved in later stages of angiogenesis (Wei et al., 2005). By recognizing the importance of the integrity of the neurovascular unit and the multiple protective effects of EPO in the ischemic brain, the present investigation examined the hypothesis in neonates that rhEPO post-ischemia treatment not only protected neuronal cells as shown before (Wei et al., 2006), but also protected vascular ECs as a mechanism of repairing the neurovascular unit after ischemic stroke. The effects of rhEPO was tested in an ischemia-only neonatal model of whisker-barrel cortex stroke that mimics common clinical cases of small ischemic strokes (Wei et al., 2006).
Materials and Methods

Animal model

Neonatal Wistar rats were randomly divided into 3 groups: 1) no-stroke control, 2) saline control, and 3) rhEPO treatment. The surgical procedure of whisker-barrel cortex ischemia in neonatal rats followed similar methods as described previously (Wei et al., 2006). Briefly, postnatal day 7 (P7) pups were anesthetized by hypothermia. Hypothermia anesthesia was chosen because many of the drugs used to anesthetize adult animals provided inadequate anesthesia for neonates or were associated with problems, such as excessively high mortality (Danneman and Mandrell, 1997). In this regard, hypothermia (immersion in ice) has been judged as a humane, safe and effective anesthesia method for survival surgeries of neonatal rats (Danneman and Mandrell, 1997). The hypothermia procedure was kept the same for all pups in different experimental groups. Pups were placed in a noninvasive head-holder to allow for a 2.5- to 3.0-mm-diameter craniectomy through the right parietal skull. The transparent dura was left intact over the whisker-barrel cortex area. Multiple branches of the middle cerebral artery (MCA) were permanently ligated with sterile no. 11 silk sutures that were passed through the dura under a dissecting microscope. This was combined with permanent cauterization of the common carotid artery (CCA). Animal care and handling followed institutional guidelines in accordance with NIH standards.

rhEPO and BrdU (5-bromo-2’-deoxyuridine) administration

rhEPO (5,000 U/kg, volume 0.10 ml, i.p., Amgen Inc., Thousand Oaks, CA) or saline (volume 0.10 ml, i.p.) was administered 60 min after ischemia and once daily after surgery for 3 days. Animals were given i.p. injections of BrdU (50 mg/kg, Sigma, St.
Louis, MO) daily, beginning on P7 until the day of sacrifice. Animals were anesthetized by an inhaled overdose of isoflurane and sacrificed at various time points following surgery.

**TUNEL staining**

The DeadEnd™ Fluorometric TUNEL System kit (Promega, Madison, WI) was used as previously described (Whitaker et al., 2007). Briefly, fresh frozen brains were mounted and sectioned in a cryostat vibratome (14 μm, Ultrapro 5000, Vibratome, St. Louis, MO). Sections were fixed in 10% buffered formalin (Fisher Scientific, Pittsburgh, PA), washed in PBS, and processed as recommended by the company’s protocol.

**Immunohistochemistry**

Frozen brain sections were fixed in 10% buffered formalin (10 min, Fischer Scientific). Immunohistochemistry proceeded as previously described (Whitaker et al., 2007). After blocking in 1.0% fish gelatin (Sigma) the slides were incubated overnight at 4 °C with the primary antibody rabbit anti-GLUT-1, mouse anti-NeuN (1:400-1:1000, AB1340, MAB377, Chemicon, Temecula, CA), or rat anti-BrdU (1:800, Abcam, Cambridge, UK). Slides were washed and incubated with secondary antibody Alexa Flour 488 anti-rabbit, anti-mouse IgG, Cy5-conjugated anti-mouse IgG, or Cy3-conjugated goat anti-rat IgG (1:200-1:1000, Molecular Probes, Carlsbad, CA), washed in PBS, and mounted with ProLong AntiFade (Molecular Probes). Slides were visualized by fluorescent and confocal microscopy (BX61, Olympus, Japan).

**Western blot**

The penumbra region of the brain was removed and frozen. Lysis buffer with protease inhibitor was added and samples were prepared with a dounce homogenizer,
centrifuged at 14,000 rpm at 4 °C for 25 min, and supernatant was collected. Protein concentration was determined by the BCA protein assay kit (Pierce Chemical Co., Rockford, IL). Proteins were separated by SDS-PAGE and transferred to a PDVF membrane in a Hoefer Mini-Gel system (Amersham Biosciences, Piscataway, NJ). Membranes were blocked in 7% nonfat-evaporated milk (Carnation, Nestle USA, Solon, OH) diluted in Tris-buffered saline, 0.1% Tween-20 at pH 7.4 (TBST) and incubated overnight at 4 °C with a primary antibody against Tie-1, Ang-2, VEGF, or bFGF (1:500-1:4000, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) diluted in 1% BSA. Mouse β-actin (1:4000, Sigma) was used for loading controls. Blots were then washed in TBST and incubated with alkaline phosphatase-conjugated anti-mouse or anti-rabbit IgG (1:5000, Promega) diluted in 3% milk TBST, washed, and the signal was detected using alkaline phosphatase substrate BCIP/NBT (Sigma). Western blotting was performed at least three times in order to confirm data reproducibility and for quantification.

**Cell counting**

For each group at least five randomized animals were used. For each animal at least three sections were randomly selected and counted. Coronal sections chosen were at least 70 µm apart. In each section, at least six random fields in the penumbra were scanned at 40x magnification. EC death in the penumbra region of coronal brain sections was evaluated by counting the number of GLUT-1-positive vessels overlapped with TUNEL staining. Angiogenesis and neurogenesis in the same region were evaluated by counting the number of GLUT-1-positive vessels co-stained with BrdU and the number of NeuN/BrdU co-labeled cells, respectively. To quantify the proximity of NeuN-positive cells to proliferating microvessels, a randomly selected GLUT-1/BrdU co-labeled
microvessel was chosen from each coronal section mentioned above, and the number of NeuN-positive cells within an 80x80 µm area was counted. Counting was aided by ImageJ image analysis system (NIH, Bethesda, MD). All counting assays were performed under blind condition.

**Statistics**

Data was graphed using GraphPad Prism, version 4 (GraphPad Software, Inc., San Diego, CA). All analyses were performed using SAS, version 9.1 (SAS Institute Inc., Cary, NC) as previously described (Whitaker et al., 2007). As a result from the hierarchical nested experimental design, the data were clustered. To properly account for this correlation, a Poisson regression using Generalized Estimating Equations (GEE) was used with the natural log of the count data for each slide, clustering on rat and slide within rat; counts within a slide were considered approximately independent of one another, while slides within a rat were considered to be correlated. This correlation was accommodated by the model. An alpha level of 0.05 was used for all counts. Data are presented as mean ± SEM. Experimental/animal numbers are reported in corresponding figure legends.
Results

**rhEPO protected against endothelial cell death**

TUNEL-positive cells are prominent in the core and penumbra regions 24 hrs after ischemia (Fig. 1A-1C). In this study, we focused on cell death and regeneration in the penumbra region. Cell death in the saline control group peaked at 24 h, and stayed at a slightly lower level over 48–72 hrs after ischemia (Fig. 1G). Administration of rhEPO (5,000 U/kg, i.p., 1, 24, 48, and 72 hrs after ischemia; same for the following experiments) significantly decreased TUNEL-positive cells in the penumbra region, although there seemed to be a cell death peak around 48 hrs after ischemia (Fig. 1G).

Colocalization of TUNEL and GLUT-1, a marker for cerebral microvessel ECs, indicated injured ECs (Fig. 1D-F) (Li et al., 2007; Whitaker et al., 2007). In rhEPO-treated animals 12-72 hrs after stroke, there were significantly fewer GLUT-1/TUNEL positive vessels compared to that in saline controls (Fig. 1H).

**rhEPO modulated expression of neurovascular regulatory proteins**

To detect rhEPO-mediated potential effects on cell responses to ischemia, protein expression of several key factors in neurogenesis and angiogenesis/vascular proliferation was investigated. Western blotting revealed significantly increased expression of Tie-1, Ang-2, and bFGF in rhEPO-treated ischemic pups (Fig. 2). There was a consistent trend of VEGF increase in the rhEPO-treated group (Fig. 2), while no change was found in the expression of Tie-2 and NGF (data not shown).

**rhEPO enhanced neurogenesis, vasculogenesis and repair of the neurovascular unit**

The focal ischemic insult reduced total neurons labeled by the specific marker NeuN in the penumbra region 7-21 days after stroke compared to no-stroke control
animals (Fig. 3A). In rhEPO-treated pups, the number of NeuN-positive cells remained stable for up to 21 days after ischemia (Fig. 3A).

To focus on post-ischemia tissue repair, we examined cell proliferation by identifying BrdU-positive cells in pups that received daily BrdU injections after stroke. Focal ischemia significantly enhanced the number of BrdU-positive cells in the penumbra region when compared with no-stroke animals (Fig. 3B). Administration of rhEPO increased the total number of BrdU-positive cells for at least 14 days after ischemia (Fig. 3B). The EPO effect subsided by the 21 days after stroke (Fig. 3B).

To elucidate whether the increased BrdU-positive cells included naïve neurons, neurogenesis was identified by the co-staining of BrdU and NeuN (Fig. 3C and 3D). Colabeled BrdU/NeuN cells in no-stroke pups were detected at a low level (Fig. 3E). Ischemia elevated the neurogenesis activity, but the activity subsided 21 days after ischemia (Fig. 3E). In ischemic pups that received rhEPO, there was a much greater increase in the number of BrdU/NeuN-positive cells; the elevation of neurogenesis remained at 21 days after ischemia when neurogenesis in the saline group declined back to a low level (Fig. 3E).

Among other cells, EC proliferation can be demonstrated by BrdU incorporation into endothelial cells marked by specific marker GLUT-1 (Wei et al., 2006; Whitaker et al., 2007) (Fig. 4 and 5). Immunohistochemical staining allowed for analysis and counting of proliferating microvessels (angiogenesis) (Fig. 4). Although ischemia stimulated proliferation of total cells (see Fig. 2), the ischemic insult in fact hampered proliferating activity of ECs (Fig. 4A-C and 5). In contrast, 7 to 21 days after ischemia the rhEPO group showed increased vessel proliferation compared with saline controls.
The largest increase was seen 14 days after ischemia. The increases in BrdU-positive vessels were consistent with a steady increase in microvessels labeled with GLUT-1 in the region 7–21 days after ischemia (Fig. 5B).

In an effort to elucidate a possible effect of rhEPO on repairing the neurovascular unit, we specifically examined the relative proximity of NeuN-positive cells to proliferating vascular ECs in the post-ischemic penumbra region. Immunohistochemical fluorescent imaging and confocal imaging confirmed that rhEPO-treated animals had significantly more NeuN-positive cells located proximal to proliferating microvessels when compared to saline controls (Fig. 5C).
Discussion

It is important to study therapeutic efficacies of potential treatments in both adult and neonatal models of stroke (Derugin et al., 2000; Northington, 2006). Studies have shown that particular treatments exert different results when comparing neonatal and adult models. Comparing a neonatal HI and an adult MCAO model, Cimino et al found that treatment with simvastatin to prevent ischemic damage only protected neonatal ischemic damage when given as a pretreatment, whereas the statin was protective in the adult model at all tested application times (Cimino et al., 2005). Moreover, statin-dependent activation of eNOS was found only in the adult MCAO model (Cimino et al., 2005). Comprehensive reviews investigating the differences between the adult and neonatal brain and stroke should be referenced for further clarification (Johnston et al., 2001; Northington, 2006).

Experiments with rhEPO treatment in stroke models have shown a multifaceted role of rhEPO on neuroprotection, neurogenesis, angiogenesis, and anti-inflammatory responses. The neuroprotective effect of EPO has been well documented in previous investigations (Demers et al., 2005). While many pathways have been implicated in rhEPO-afforded neuroprotection, fewer studies have defined a role for rhEPO in vascular protection in vivo. The present investigation demonstrates in neonatal ischemic stroke that at a clinically relevant dosage administered 60 min after ischemia rhEPO is not only protective against neuronal cell death, but also shows significant protection of vascular ECs. As a result, rhEPO increases the number of neurons and microvessels from days to several weeks after stroke. In addition, rhEPO stimulates neurogenesis and angiogenesis/vasculogenesis, likely mediated by increased expression of several key
molecules in these processes. The increased distribution of newly formed neurons close to ECs suggests an enhanced repair process of the neurovasculature or the neurovascular unit. This orchestrated regeneration of vascular ECs and neuronal cells may serve as an important mechanism of rhEPO-mediated therapeutic effects. In the present investigation, rhEPO was administrated for 3-4 days, which is unlikely to trigger a hematopoietic effect; in humans, for example, stimulation of hematopoietic response is usually achieved after several weeks of rhEPO administration (Duhrsen and Hossfeld, 1994).

We have previously demonstrated that rhEPO significantly attenuated cell death, specifically neuronal cell death, in the same model of neonatal whisker-barrel cortex stroke (Wei et al., 2006). This neonatal mini-stroke model presents with an initial necrotic component of cell death followed with primary features of apoptosis as neuronal injury induced by focal ischemia (Wei et al., 2006). With the advent of the phenomenon of neurovascular unit, the inextricable relationship between neurons and the vasculature is becoming apparent. Hypoxic conditions stimulate expansion of the microvascular system in the brain (Ogunshola et al., 2000). The EPO-mediated protective effect on ECs was shown in vitro where increased numbers of ECs was due to an increase in proliferation and reduced apoptosis (Muller-Ehmsen et al., 2006). We hypothesized that rhEPO treatment would have a similar effect on vascular proliferation in vivo. Increased GLUT-1-positive microvessels were confirmed after rhEPO treatment, likely resulting from rhEPO-mediated protective and proliferative effects. We observed an overall increase in cell proliferation after rhEPO administration. Seven and 14 days after ischemia there was significantly more cell proliferation in the rhEPO group compared to controls, suggesting a period of active regeneration in the ischemic and surrounding area.
An interesting observation is that while rhEPO enhanced neurogenesis (BrdU/NeuN positive cells) and angiogenesis (BrdU/GLUT-1 positive cells), the total BrdU positive cells increased at 14 days but not 21 days after stroke in the rhEPO-treated group (Fig. 3B). Two possibilities may most likely explain the observation: 1) EPO neuroprotection in the neonate has been shown to involve the blocking of a secondary delayed rise in interleukin-β and other inflammatory mediators (Sun et al., 2005). Proliferation of non-neuronal cells such as those in inflammatory reactions may be prohibited by rhEPO at this time; 2) subsided effect of rhEPO due to the short administration time (4 days).

Normal neonatal angiogenesis peaks between P13-P24, stabilizes between P24–P33, and decreases after stability (Ogunshola et al., 2000). The neonatal controls in our study followed a similar trend, as did the rhEPO-treated group. We observed an increase in microvessel proliferation 7 and 14 days following stroke (P14 and P21) after rhEPO treatment and a similar decrease and stabilization of angiogenesis by 21 days after ischemia (P28) (Fig. 4 and 5). While BrdU proliferation in the saline control group was observed, this did not translate into increased microvessels. The post-ischemia brain may have been too harsh an environment for successful vascular regeneration, which suggests the importance of a protective as well as regenerative therapy after stroke.

The concept of the neurovascular unit led us to investigate angiogenesis/vascularization and neurogenesis. rhEPO treatment resulted in an increase in neurogenesis 7 to 21 days after stroke in the rhEPO group. ECs secrete soluble factors that stimulate the self-renewal of neural stem cells in the vascular niche (Shen et al., 2004). Administration of human cord blood-derived CD34+ cells after stroke induced
angiogenesis which led to secondary neurogenesis (Taguchi et al., 2004). Our study may support the idea that an angiogenic response results in the establishment of a favorable environment for the regeneration of neurons (Taguchi et al., 2004). We observed an increase in the number of neurons in close proximity to angiogenic microvessels in the rhEPO-treated animals (Fig. 4G and 5C). EC and neuronal proliferation and viability intimately affect each other. Angiogenic and neurogenic factors are important in forming and maintaining a functional neurovascular system. In the present investigation, both processes increased between 7 and 21 days after ischemia, further implying that this is the period of high proliferative activities and a potential target for therapeutic treatments. Recently, Ang-1 and Tie-2 have been implicated to mediate neuroblast migration to the penumbra region following adult mouse stroke (Ohab et al., 2006). The intimate and complicated relationship between the angiopoietins and the corresponding receptor tyrosine kinases Tie-1 and Tie-2 allows for the possibility that this system may be having an effect in progenitor proliferation and differentiation in our model of rhEPO-mediated neurovascular remodeling following neonatal focal ischemia. Furthermore, as previously stated, bFGF and various FGF receptors have also been implicated in neural progenitor modulation (Mudo et al., 2007; Xiao et al., 2007). While we have not determined precisely where the neural and vascular progenitor cells are derived from in our model, we presume that a majority of the cells come from the subventricular zone. The simultaneous effect of rhEPO on protection and subsequent proliferation may, in turn, allow for a neurogenic and angiogenic effect that is modulated by the expression of the Tie/Ang system and/or bFGF. The nurtured brain microenvironment that results from
rhEPO administration may further promote repair of the neurovascular unit in the penumbra region, thus eventually translating to potential functional recovery.

EPO and EPOR are also expressed in glial cells and their expression increases following cerebral ischemia (Bernaudin et al., 1999). rhEPO markedly reduces astrocyte activation, the recruitment of leukocytes and microglia into ischemic area, and the production of the proinflammatory cytokines in the ischemic rat brain (Villa et al., 2003). These events should benefit the protection and repair of the neurovascular unit, although one study reported that EPO (30 pM) in cortical cultures did not show protective effects against hypoxia- or AMPA-induced glial cell death (Sinor and Greenberg, 2000).

Our study appears to be the first to investigate the effect of rhEPO on vascular protection and neurovasculature repair in an in vivo neonatal stroke model. rhEPO has recently emerged as a hopeful neuroprotective drug. We now present results that in addition to neuroprotection, rhEPO is also protective against ischemia-induced EC death in the neonatal brain. The multifaceted effects of rhEPO, including cell protection, neurogenesis, and angiogenesis, are essential for preservation of the neurovascular unit that is the fundamental element of the brain circulation and neuronal activities. The possibility that EPO may show similar comprehensive effects in adult animals remains to be tested.
References


Footnotes

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Legends for Figures

Figure 1. rhEPO protected against endothelial cell death.

Treatment of stroke with rhEPO (5,000 U/kg, i.p., 1, 24, 48, and 72 hrs after ischemia) decreases cell death in the penumbra region. Frozen sections were stained 12-72 hrs after ischemia for cell death assay. **A.** TUNEL staining (green) depicted cell death and DNA damage in the ischemic core (*) and penumbra (dotted lines). GLUT-1 (red) and NeuN (blue) staining revealed microvessels and neurons, respectively. **B and C.** Twenty four hrs after insult, saline control ischemic animals (B) showed more TUNEL/GLUT-1 positive vessels when compared to that in rhEPO treated rats (C). Arrows indicate TUNEL and GLUT-1 colocalization. **D-F.** Confocal 3D images show a GLUT-1-positive microvessel (D), TUNEL-positive cells (E), and the merged image of a TUNEL/GLUT-1-positive endothelial cell (arrow indicates colocalized area) (F). **G.** rhEPO treatment decreased cell death after stroke. At 24 and 72 hrs, the rhEPO-treated group exhibited less cell death although no significant difference was seen at 48 hrs between the saline control and rhEPO-treated group. Cell death in no-stroke controls was negligible. **H.** Quantification of EC death (TUNEL/GLUT-1-positive) 12-72 hrs after ischemia. Treatment with rhEPO markedly reduced EC death at all time points tested. EC death in no-stroke controls was negligible. N≥6 animals per group; *. P<0.05 compared to saline control.

Figure 2: rhEPO modulated the expression of neurovascular regulatory proteins.

The protein levels of Tie-1, Ang-2, bFGF, and VEGF were detected using Western blot analysis. Representative electrophoresis gels show the levels of angiogenic
factors 7-21 days after ischemia with and without rhEPO treatment (5,000 U/kg, i.p., 1, 24, 48, and 72 hrs after ischemia). A-D. Gray intensity quantification for comparisons of each factor is shown. Blots were normalized against β-actin and quantified using ImageJ software. rhEPO administration enhanced the expression of (A) Tie-1 (14 days), (B) Ang-2 (7 and 21 days), and (C) bFGF (7 to 21 days). There was a consistent trend of increased (D) VEGF expression in rhEPO treated animals. N≥3 animals for each test; *, P<0.05 compared to saline controls.

Figure 3: rhEPO enhanced neurogenesis.

Neurogenesis was examined by the colocalization of the neuronal marker NeuN and the proliferation marker BrdU. A. Focal ischemia reduced the number of NeuN-positive cells in the penumbra region 7-21 days after stroke. At 14 days there were significantly more NeuN-positive cells in the rhEPO group compared to saline controls. B. To label proliferating cells, BrdU (50 mg/kg) was administered once a day following stroke. Seven to 14 days after ischemia, BrdU staining revealed a significant increase in cell proliferation in rhEPO-treated animals when compared to controls. By 21 days, the rhEPO effect on cell proliferation declined. C. NeuN (green) and BrdU (red) staining showed newly developed neurons. D. Confocal image of NeuN and BrdU colocalization, enlarged 3-D image from the frame in C. E. Summarized data on neurogenesis. At 7 and 21 days after stroke the number of NeuN/BrdU-positive cells was significantly more in the rhEPO group compared to saline controls. N≥6, *, P<0.05 compared to saline controls, †, P<0.05 compared to no-stroke controls.
Figure 4: rhEPO enhanced angiogenesis.

Angiogenesis was examined by the colocalization of EC marker GLUT-1 with BrdU staining. A–C. Representative immunohistochemical staining of BrdU (red) and GLUT-1 (green) 14 days after ischemia in no stroke control, saline control, and rhEPO-treated groups. D. Low magnification of the penumbra region stained for BrdU (red), GLUT-1 (green), and NeuN (blue). Arrows point to proliferating microvessels. E. Higher magnification and confocal 3D image of proliferating vessel in D indicative of angiogenesis in the penumbra region after stroke. F. 200x image of part of microvessel in E. G. Confocal 3D image of the colocalization of BrdU and GLUT-1 staining (arrows) indicative of a proliferating microvessel. The blue arrow denotes a BrdU and NeuN colocalized cell in close proximity to the microvessel. H. The same confocal 3D image is shown with only the Cy3 channel to clearly show the BrdU positive cells. I. Confocal grid image of the proliferating vessel seen in G.

Figure 5: rhEPO and neurovascular repair.

A. 7-21 days following stroke there was a significant increase in angiogenesis in the rhEPO treatment group when compared to saline controls. B. Treatment with rhEPO increased the number of GLUT-1-positive vessels in the penumbra region 7-21 days after ischemia. C. The general proximity of NeuN-positive neurons to BrdU/GLUT-1 colocalized microvessels was assessed 14 days after stroke. There were significantly more neurons associated with microvessels within the area of an 80 x 80 μm grid in the rhEPO-treated group compared to saline controls. N≥6 animals per group; *. P<0.05 compared to saline controls, †. P<0.05 compared to no-stroke controls.
Figure 1.
Figure 2.

A

Tie-1

B-actin

Gray Intensity

Saline Control

rhEPO Treatment

7 days

14 days

21 days

B

Ang-2

B-actin

Gray Intensity

Saline Control

rhEPO Treatment

7 days

14 days

21 days

C

bFGF

B-actin

Gray Intensity

Saline Control

rhEPO Treatment

7 days

14 days

21 days

D

VEGF

B-actin

Gray Intensity

Saline Control

rhEPO Treatment

7 days

14 days

21 days
Figure 3.
Figure 4.
Figure 5.

A

BrdU positive vessels/field

- No Stroke
- Saline Control
- rhEPO Treatment

7 days 14 days 21 days

B

GLUT-1 positive vessels/field

- No Stroke
- Saline Control
- rhEPO Treatment

7 days 14 days 21 days

C

NeuN-positive cells

- Saline Control
- rhEPO Treatment

80 x 80 mm