Neurobiology of Disease 93 (2016) 57–63

Contents lists available at ScienceDirect

Neurobiology of Disease

journal homepage: www.elsevier.com/locate/ynbdi

Delayed erythropoietin therapy improves histological and behavioral outcomes after transient neonatal stroke

Amara Larpthaveesarp [a], Margaret Georgevits [a], Donna M. Ferriero [a,b], Fernando F. Gonzalez [a,*]

[a] Department of Pediatrics, University of California San Francisco, San Francisco, CA 94143, United States
[b] Department of Neurology, University of California San Francisco, San Francisco, CA 94143, United States

ARTICLE INFO

Article history:
Received 6 October 2015
Revised 6 April 2016
Accepted 29 April 2016
Available online 30 April 2016

Keywords:
Neonatal stroke
Ischemia
Erythropoietin
Brain injury
Neuroprotection

ABSTRACT

Background and purpose: Stroke is a major cause of neonatal morbidity, often with delayed diagnosis and with no accepted therapeutic options. The purpose of this study is to investigate the efficacy of delayed initiation of multiple dose erythropoietin (EPO) therapy in improving histological and behavioral outcomes after early transient ischemic stroke.

Methods: 32 postnatal day 10 (P10) Sprague-Dawley rats underwent sham surgery or transient middle cerebral artery occlusion (tMCAO) for 3 h, resulting in injury involving the striatum and parieto-temporal cortex. EPO (1000 U/kg per dose × 3 doses) or vehicle was administered intraperitoneally starting one week after tMCAO (at P17, P20, and P23). At four weeks after tMCAO, sensorimotor function was assessed in these four groups (6 vehicle-sham, 6 EPO-sham, 10 vehicle-tMCAO and 10 EPO-tMCAO) with forepaw preference in cylinder rearing trials. Brains were then harvested for hemispheric volume and Western blot analysis.

Results: EPO-tMCAO animals had significant improvement in forepaw symmetry in cylinder rearing trials compared to vehicle-tMCAO animals, and did not differ from sham animals. There was also significant preservation of hemispheric brain volume in EPO-tMCAO compared to vehicle-tMCAO animals. No differences in ongoing cell death at P17 or P24 were noted by spectrin cleavage in either EPO-tMCAO or vehicle-tMCAO groups.

Conclusions: These results suggest that delayed EPO therapy improves both behavioral and histological outcomes at one month following transient neonatal stroke, and may provide a late treatment alternative for early brain injury.

1. Introduction

Stroke during the neonatal period is a significant cause of death and long-term disability, occurring in as many as 1 in 2300 live births (Grunt et al., 2015). Most survivors have long-term motor or cognitive dysfunction, yet despite these lifelong effects no accepted post-injury treatment exists. In addition, many cases are identified outside of the perinatal period, which further complicates therapeutic strategies as effective treatments initiated remotely from the insult would be necessary to benefit many affected infants.

It is clear that injury continues to progress over a period of days to weeks following the primary insult (van der Aa et al., 2013). This involves a variety of mechanisms and pathways that result in early necrosis and later programmed cell death, as well as decreased cell proliferation and altered cell fate. Of many potential therapies that have been studied in an effort to both suppress early cell death but also enhance later proliferation and repair, erythropoietin (EPO) has shown promise in a number of brain injury models. EPO is a pleiotropic cytokine with a number of erythropoietic and non-erythropoietic roles (Wu and Gonzalez, 2015). EPO and EPO receptor (EPO-R) expression are elevated in the brain during gestation but decline rapidly after birth, with cell-specific endogenous EPO/EPO-R upregulation after injury (Bernaudin et al., 1999). Following hypoxia there is stabilization of HIF-1, with increased expression of downstream targets and growth factors that include EPO and VEGF (Bernaudin et al., 2002; Mu et al., 2005). This results in specific expression of EPO and its receptor on neurons, astrocytes and microglia at different time points that initiate endogenous mechanisms for neuroprotection and repair (Bernaudin et al., 1999). Initiation of these intracellular processes lead to anti-apoptotic, anti-inflammatory and pro-angiogenic effects, and play a significant role in neurogenesis and cell fate outcome (Xiong et al., 2011).

We have previously described a non-hemorrhagic ischemia-reperfusion stroke model in the immature rat using transient middle cerebral artery occlusion (tMCAO) (Derugin et al., 1998; Gonzalez et al., 2013). This is similar to the most common cause of stroke in the perinatal period (Rutherford et al., 2012; van der Aa et al., 2014). We have demonstrated increased cell proliferation and migration from the subventricular zone (SVZ), with altered cell fate favoring newly born

* Corresponding author at: 1550 4th St, Room RH-382C, Campus Box 2711, San Francisco, CA 94158, United States.
E-mail address: Fernando.Gonzalez@ucsf.edu (F.F. Gonzalez).
Available online on ScienceDirect (www.sciencedirect.com).

http://dx.doi.org/10.1016/j.nbd.2016.04.006
0969-9961/

neurons and oligodendrocyte precursors in the injured brain following EPO treatment (Gonzalez et al., 2013). Single dose EPO therapy given immediately following tMCAO preserved short-term histological and sensorimotor outcomes (Chang et al., 2005; Gonzalez et al., 2007), while three doses administered over a 1-week period were required for long-term improvement in both histologic brain volume and cognitive function (Gonzalez et al., 2009).

Identification and diagnosis of perinatal stroke is often delayed in neonates, who initially present with seizures (Grunt et al., 2015). Given the anti-apoptotic, pro-angiogenic and neurogenic effects of EPO in ischemia models, and the knowledge that the injury continues to evolve beyond the acute phase of ischemia, the question arises regarding the benefits of EPO initiated at more remote time periods. While multiple dose EPO has demonstrated long-term benefit in this model when initiated immediately following occlusion, and hypothermia has demonstrated benefit for hypoxic-ischemic injury within a tight therapeutic window (Tagin et al., 2012), late treatment alternatives for ischemic brain injury do not exist. For this reason, we examined the efficacy of a three dose exogenous EPO regimen initiated at one week following stroke, hypothesizing that treatment starting at a late time point would still have significant histological and functional benefit.

2. Materials and methods

The protocol for this study received approval from the University of California, San Francisco Institutional Animal Care and Use Committee, and all studies were conducted in accordance with the United States Public Health Service's Policy on Humane Care and Use of Laboratory Animals. Every effort was made to minimize animal suffering and to reduce the numbers of animals used.

2.1. Transient middle cerebral artery occlusion

Postnatal day 10 (P10) Sprague-Dawley rats, each weighing 19–21 g, underwent focal ischemia-reperfusion with right transient middle cerebral artery occlusion (tMCAO) for 3 h, or sham surgery (Derugin et al., 1998; Gonzalez et al., 2013). This age was chosen to approximate development of the full-term human newborn brain (Hagberg et al., 2002). Female rats with 7-day old litters (approximately 10 pups per litter) were purchased from Simonson Labs (Gilroy, CA, USA). Mothers were housed in a temperature and light-controlled facility and given ad libitum access to food and water until pups were 10 days old. tMCAO or sham surgery was performed in spontaneously breathing animals anesthetized with 3% isoflurane in 100% O_2. Following induction, rectal temperature was monitored and maintained at 36 °C–37 °C with a combination of heating blanket and overhead light until recovery from anesthesia. The right internal carotid artery (ICA) was dissected and a temporary ligature was tied using a strand of 6-0 suture at its origin. This ligature was retracted laterally and posteriorly to prevent retrograde blood flow. A second suture strand was looped around the ICA above the pterygopalatine artery and an arteriotomy was made proximal to the isolated ICA. A silicone coated 6-0 nylon filament from Doccol Corporation (Sharon, MA, USA) was inserted 9–10.5 mm (based on animal weight) to occlude the MCA and the second suture strand was tied off to secure the filament for the duration of occlusion. Following recovery from anesthesia, pups were returned to their dam for the duration of the occlusion. Injury was confirmed by severe left frontal/hindlimb paresis resulting in circling movements during the occlusion period. We have previously demonstrated a consistent pattern of injury involving the striatum and parieto-temporal cortex with this model using MRI during occlusion and TTC staining at 24 h following tMCAO (Gonzalez et al., 2013; Gonzalez et al., 2007; Gonzalez et al., 2009). For reperfusion, each animal was anesthetized and all suture ties and the occluding filament were removed. Avitene microfibrillar collagen hemostat (Warwick, RI, USA) was placed over the arteriotomy and the skin incision was closed. Sham animals were anesthetized and the ICA was dissected, after which the skin incision was closed. At the time of reperfusion, the sham animals were once again anesthetized for 5 min, equivalent to the reperfusion procedure time for tMCAO animals. 20 animals underwent tMCAO and 12 animals received sham surgery. Animal sex was equally distributed amongst the four groups, and there were no deaths.

2.2. Erythropoietin treatment

Rats were treated with intraperitoneal (IP) doses of EPO (1000 U/kg) at three time points: 7 days (P17), 10 days (P20), and 13 days (P23) after injury. Sham animals received vehicle (0.1% BSA) IP at these same time points. Weight was monitored for one week following tMCAO or sham surgery to ensure adequate weight gain. There were four experimental groups: vehicle-sham (n = 6), EPO-sham (n = 6), vehicle-tMCAO (n = 10), and EPO-tMCAO (n = 10).

2.3. Behavioral testing

Cylinder rearing was used to assess the effects of ischemic injury and EPO treatment on forelimb use as a function of sensorimotor bias. Animals with unilateral ischemic brain injury exhibit forelimb preference shown by favoring use of the non-impaired limb for touching or bracing the side of the cylinder, and rats are capable of exploring the walls of the cylinder as early as P21 (Grow et al., 2003). Forelimb movements for each rat were analyzed during exploratory activity in a transparent Plexiglas cylinder measuring 20-cm in diameter and 30-cm in height in two trials conducted on consecutive days at ~4 weeks after tMCAO (P37 and P38). The size of the cylinder allowed free movement but was small enough to encourage exploration and touches/braces onto the side of the cylinder, while its height prevented the rat from reaching the top edge and its heavy weight prevented its movement during braces. Animals were handled for about 5 min per day for three days prior to testing. Each animal was then individually placed in the cylinder in a quiet room without distinctive markings and observed for 3 min in each trial, with results averaged per animal. Initial forepaw placement of each weight-bearing contact with the wall was recorded as right, left, or both forepaws (Gustavsson et al., 2005). Results were expressed as the percentage use of the non-impaired (right) forepaw for braces relative to the total number of forepaw initiations. The results were analyzed by two independent raters and the average scores of the two raters blinded to group were used for data analysis.

2.4. Histology

Immediately following behavioral testing at P38, animals were anesthetized with sodium pentobarbital (100 mg/kg; Nembutal, Abbott Labs, Abbott Park, Ill., USA) and sacrificed. Brains were harvested by transcardiac perfusion with 4% paraformaldehyde (PFA) in 0.1 M phosphate-buffered saline (pH 7.4). Brains were carefully removed and postfixed overnight, equilibrated in 30% sucrose in 0.1 M PBS and left at 4 °C in 0.1 M PBS until sectioning and staining. The olfactory bulbs and cerebellum were removed, and the entire brain was sectioned at 50-μm intervals on a sliding microtome (Thermo Scientific, Waltham, MA, USA). The mounted sections were air-dried, stained with cresyl violet, dehydrated in graded ethanol solutions, cleared in Citrisolv (Fisher Scientific, Pittsburgh, PA, USA) and cover slipped in Permount (Fisher Scientific).

2.5. Stereological volumetric analysis of brain volumes

Using systematic random sampling, a series representing every 12th section was selected, cresyl violet stained, and analyzed. Sections encompassed the whole brain rostrally from the genu of the corpus callosum through the posterior portion of the hippocampus to the