Research article

Erythropoietin-mediated neuroprotection in a pediatric mouse model of chronic hypoxia

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HIGHLIGHTS

• Pediatric mouse model of chronic hypoxia decreases the hippocampal progenitor pool.
• Pediatric mouse model of chronic hypoxia decreases the oligodendrocyte progenitors.
• Erythropoietin rescues the decreased hippocampal progenitor pool.
• Erythropoietin rescues the decreased oligodendrocyte progenitor pool.

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ABSTRACT

Chronic hypoxia (CH), a disease state that accounts for significant morbidity and mortality in pediatrics, occurs in many children during critical periods of hippocampal development and cortical myelination. Hippocampal neurogenesis occurs throughout postnatal life and is important for normal development, thus impairment results in long-term cognitive deficits. Erythropoietin (EPO), a drug commonly known for its role in erythropoiesis, has recently been evaluated in neuroprotection in neonatal injury models and preterm brain injury. However, the effects of EPO therapy on hippocampal neurogenesis and myelination in pediatric CH are unknown. We show that CH decreases hippocampal neurogenesis in a pediatric mouse model. This decrease in early and late progenitors, and actively dividing cells is rescued with EPO treatment. Furthermore, we show that CH during this critical time decreases oligodendrocyte progenitor (OPC) populations in the cortex, leading to defective myelination. However, EPO therapy is only able to rescue the OPC but not the loss of mature myelin. Overall, our findings demonstrate that CH in developing mice has significant effects on hippocampal neurogenesis and OPCs, which can be rescued with EPO treatment. Future studies should confirm the role of this FDA-approved therapy in neuroprotection in at-risk pediatric populations.

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1. Introduction

CH accounts for significant morbidity and mortality in pediatrics [1]. CH in pediatrics occurs at two important time periods; neonatal period, and outside of the neonatal period. Much of the research to date on CH in pediatrics has focused on the neonatal period, including congenital heart disease and premature infants [2]. However, CH outside of the neonatal period accounts for a significant disease burden with lung disease, such as acute respiratory distress syndrome, pulmonary hypertension, and cystic fibrosis as well as in patients with obstructive sleep apnea [3].

Distinct regions of the brain, including the hippocampus, exhibit a high sensitivity to hypoxia [4]. The hippocampus, located in the medial temporal lobe, is critical in consolidation of short-term memory into long-term memory, spatial orientation and navigation. The dentate gyrus, is one of two brain regions in the hippocampus that exhibit postnatal neurogenesis [5]. Dentate gyrus neural stem cells are broadly classified into two groups: types 1 and 2a, which express neuroepithelial marker nestin and types 2b and 3, which express developmental neuronal markers, such as microtubule protein double-cortin (DCX) [6]. These cells are stimulated in response to both physiological stimuli such as exercise and
environmental enrichment and pathological stimuli including trauma [7,8].

Since CH occurs at critical time in the brain where neurogenesis is followed by generation of OPC, which form mature myelin, it can also disturb OPC maturation. This results in disrupted myelination and subsequent white matter injury such as periventricular leukomalacia [9]. OPCs represent a variety of neuroglia that are present exclusively in the central nervous system and aid with the myelination/insulation of axons to augment impulse propagation [10]. In rodent models, OPCs arise during late embryogenesis and early postnatal development, similar to neurogenesis from cells in the supraventricular zones of the lateral ventricles [11]. The cells migrate from these germinal zones to populate gray and white (myelinated) matter areas.

Erythropoietin (EPO) is a 30.4-kD glycoprotein with four carbohydrate residues, and a pleiotrophic cytokine. It is normally produced in the liver and kidney of adult mammals, though induced production and EPO receptors (EPO-R) are also seen in the brain [12]. It is most commonly known for its role in erythropoiesis. However, EPO increases neural stem cell proliferation, neurite outgrowth and neurovascular remodeling [13]. EPO therapy induces neuroprotection in animal models of spinal cord injury, traumatic brain injury, ischemic stroke and perinatal asphyxia. The clinical neuroprotective efficacy of EPO has also been studied in human preterm infants and neonates [13]. Importantly, EPO improves neurodevelopmental outcomes for preterm infants in retrospective studies at 18 months of age for term infants with moderate hypoxic ischemic encephalopathy [12]. However, there have been no studies to date on the effect of EPO in pediatric CH. Hence, we developed a pediatric CH model at a later time period in rodents with CH from postnatal day (P) 21 to P35. This time frame, in terms of brain maturation, corresponds to the pediatric age group. We hypothesized that CH would decrease hippocampal neurogenesis and impair OPC maturation in a pediatric CH mouse model and that these detrimental effects could be rescued by EPO therapy.

2. Materials and methods

2.1. Animal preparation

The Institutional Animal Care and Use Committee at the UTSW approved the study. The animals were housed in the ARC with 12-h dark light cycle. Transgenic mice that express enhanced green fluorescent protein (eGFP) under the control of the nestin promoter were used for all the experiments [14].

2.2. Experimental design

Pups were weaned from their mothers at P 21. Mice were randomly picked from 3–5 liters and separated into four groups: control group, control + EPO, CH group, and CH + EPO. CH groups were housed in a Plexiglas chamber exposed to 10% O2 ± 0.1% from P21 to P35 [14]. To determine the progenitor stem cell population, a single intraperitoneal dose of BrdU (50 mg/kg sterile H2O:

Fig. 1. Chronic hypoxia decreases actively dividing cells. Representative pictures of BrdU expression in the dentate gyrus from control, CH and CH + EPO (C and D, CH [1]-E and CH + EPO-F), respectively, showing decrease in the actively dividing cells in CH (G). p-values for BrdU between control and CH is significant **p < 0.01 (n = 7/group) and between CH and CH + EPO p = 0.057. Error bars represent standard deviation (SD) and the scale bar represents 35 μM. GL, granular layer and SGZ, subgranular zone.
Sigma–Aldrich) was given 2 h prior to sacrifice on P35. EPO groups were given daily intraperitoneal injections of human recombinant EPO at 5000 IU/kg from P21 to P35. All groups were sacrificed at P35.

2.3. Immunohistochemistry (IHC)

Animals were anesthetized, transcardially perfused, brains fixed and sectioned on a vibrating microtome (Leica Microsystems). Serial coronal 50 μM sections were obtained through the hippocampus from bregma (−1.0 to −3.4). A DAB protocol was used for stereology measurements and double-labeling immunofluorescence for images shown for neurogenesis. IHC performed as outlined in Ref. [14] for neurogenesis.

For anti-platelet-derived growth factor receptor – α (PDGFR-α) IHC, images were obtained in the Nanozoomer (Hamamatsu). Sections were blocked and incubated with primary antibodies (rat anti-PDGFR-α, 1:250; BD Pharmingen; 4 °C) overnight, washed and incubated with secondary antibodies (Cy-2, 1:200, 4 °C, 2 h) followed by 3,3′-Diaminobenzadine (DAB) amplification. For the purpose of obtaining images after incubation with secondary antibody for 2 h, Cy-3 and Cy-2 were used, 1:200 for MBP and PDGFR-α, respectively. The sections were washed, mounted and cover slipped with Immu-Mount (Thermo Scientific, Waltham, MA, USA).

2.4. Cell quantification

Unbiased estimates for cell counts were obtained using stereological quantification on Olympus BX51 System Microscope with MicroFIRE A/R camera (Optronics) and Optical Fractionator Probe (Stereo Investigator, Microbrightfield). Minimum of 350 cells per animal was counted. For OPC quantification using PDGFR-α, images obtained (Nanozoomer, Hamamatsu) and OPC’s quantified. A 500 μm² area of cortex 200 μm lateral to the midline and 200 μm above the corpus callosum (8–10/animal). Optical dissector height was 24 μm with 3 μm guard zone. The average Schaffer coefficient of Error <10%.

To reduce bias, samples were identically processed. Every 6th section was used for counting and only animals where all the sections present were used for quantification. Publication images were obtained by Zeiss LSM 510 confocal microscope utilizing Argon 488 and He 633 lasers for GFP, DCX and BrdU images.

2.5. Western blot

Cortex dissected on P35 and concentration determined by BCA protein kit using β-tubulin (Sigma) at 1:30,000 as standard aliquots (10 g protein) were separated on 15% polyacrylamide gels and transferred onto difluoride membrane. Mature oligodendrocytes (mouse anti-myelin basic protein antibody, SMI-99, 1:1000; Covance) were detected using enzyme chemiluminescence kit. To ensure equal loading and accuracy, protein levels were normalized to protein levels of Tubulin, which is a structural protein present within the cells.

2.6. Statistics

One-way ANOVA with Bonferroni correction was used to for multiple comparisons. The data are presented as mean +/- SD and statistical significance was set at p < 0.05.

3. Results

Animals in the CH group were smaller (weight in gms) compared to the control group (control 27.3 ± 2.8, control + EPO 25.8 ± 2.5 vs. CH 20.3 ± 1.4, CH + EPO 19.8 ± 1.5, p < 0.0001) with elevated HCT in CH group (control 50.2 ± 1.6 vs. CH 55.6 ± 1.9, p < 0.0001) and group treated with EPO (control 50.2 ± 1.6 vs. control + EPO 59.4 ± 4.5, p < 0.0001 and CH 55.6 ± 1.9 vs. CH + EPO 75.4 ± 8.2, p < 0.0001).

![Fig. 2. Chronic hypoxia decreases early progenitors and rescued by EPO treatment. Representative pictures of GFP expression in the dentate gyrus from control, CH and CH + EPO (Control-A, CH-B, CH + EPO-C), respectively, showing decrease in GFP expressing early progenitors in CH that is rescued by treatment with EPO (D). p-value for GFP between control and CH *p < 0.001–0.01 (n = 5–7/group) and between CH and CH + EPO **p < 0.001–0.01 (n = 5–7/group). Error bars represent SD and the scale bar represents 35 μM. GL, granular layer and SGZ, subgranular zone.](image-url)
3.1. Pediatric CH decreases neurogenesis in the dentate gyrus

We quantified the number of early, late progenitors and actively dividing cells in the nestin-GFP transgenic mice on P35. CH decreased actively dividing (BrdU-expressing) cells in the CH group by 34% (190 ± 33 in CH vs. 288 ± 39 in control group) (Fig. 1A, B, D and G). To determine whether these actively dividing cells were stem/progenitor cells that express GFP, we performed stereological counts of early progenitors and found them to be decreased in the CH group by 25% (22,808 ± 2666 in CH vs. 30,225 ± 6153 in control group) (Fig. 2A, B and D). Since there was decrease in the actively dividing cells and early progenitors we next quantified the late progenitors that were labeled by DCX expression. We determined that DCX expressing cells were also decreased by 33% in the CH group (31,312 ± 5447 in CH vs. 46,419 ± 8027 in control) (Fig. 3A, B and D).

3.2. Erythropoietin rescues neurogenesis following chronic hypoxia

Since EPO has been shown to increase neuronal stem cell proliferation in other models of injury we hypothesized that the decrease in neurogenesis seen during pediatric model of CH could be rescued by systemic administration of EPO. Following EPO injections, actively dividing cells that were decreased by 34%, after CH, were now increased by 18% though this remained insignificant compared to the untreated CH group (225 ± 36 in CH+EPO vs. 190 ± 33 in CH) (Fig. 1 F and G). EPO in the absence of CH did not affect proliferation (255 ± 50 in control+EPO vs. 288 ± 39 in control). EPO during CH additionally increased the early progenitor cell population (GFP expressing cells) by 43% (32,705 ± 3928 in CH+EPO vs. 22,808 ± 2666) in CH (Fig. 2C and D). EPO alone had no effect on the control group (30,225 ± 6153 in control + EPO vs. 27,053 ± 2356 in control). Further quantification of DCX expressing late progenitors showed a similar rescue with EPO therapy with increase by 27% (43,031 ± 3008 in CH+ EPO vs. 31,312 ± 5447) (Fig. 3C and D) in CH. There was no difference in the control group that received EPO (44,345 ± 6581 control plus EPO vs. 46,419 ± 8027 in the control).

3.3. Pediatric CH decreases oligodendrocyte progenitors and mature myelin

Pediatric CH occurs at a critical time when OPC maturation and myelination occur in the developing brain. Therefore, we assessed the effect of CH on OPC by quantifying PDGFRα-expressing OPCs in the cortex using design-based stereology at P35. CH induced a 15% decrease in the PDGFRα-expressing OPC (8123 ± 777 in CH vs. 9557 ± 343 in control) (Fig. 4A, B and E). CH induced a concomitant reduction in mature myelin as evaluated by cortical MBP protein levels at P35 (Fig. 4D and F). EPO therapy rescued CH-induced reduction in OPC population similar to the rescue of neurogenesis by effectively countering the OPC loss (8123 ± 777 in CH vs. 9459 ± 940 in CH plus EPO) (Fig. 4C and E). However, EPO did not prove efficacious in rescuing levels of mature myelin following CH.

4. Discussion

CH in the developing brain has been widely studied, but most of the research to date has focused on the neonatal populations both in animal and human studies. However CH remains a significant disease burden in the older pediatric population as well. It has been well established that CH disturbs various aspects of brain development outside of the neonatal period and there remains no effective therapy. We show that CH in our novel pediatric mouse model causes significant decrease in hippocampal neurogenesis which is not surprising as the peak of neurogenesis in the dentate gyrus occurs during the time of exposure to CH. Changes in the microenvironment around the critical time of rapid proliferation, explains the decrease in not only the early progenitors but also the late progenitors and actively dividing neuronal population.
This perturbation of hippocampal neurogenesis during pediatric CH may contribute to the cognitive delay seen in this age and should be investigated in future studies.

Erythropoietin is well established for having neuroprotective properties [13]. The genetic expression of EPO and its receptors located on most neurons can be triggered by hypoxia-inducible factor 1 (HIF-1). These are upregulated under hypoxic conditions through tightly regulated pathways [15] thereby mediating neuroprotection [15]. EPO stimulates pluripotent progenitor cells to differentiate into neural progenitor cells in vitro. Studies in the brain stem of hypoxic mice [16] as well as the cortex of mice exposed to intermittent hypoxia demonstrated increased mRNA expression of the EPO and EPO-R genes under hypoxic conditions [17]. However, our pediatric CH mouse model induced a counter-intuitive decrease in neurogenesis. This is intriguing since it has been shown that CH causes up regulation of HIF-1 with subsequent increase in EPO production and EPO receptor expression [18]. However, our pediatric CH mouse model induced a counter-intuitive decrease in neurogenesis. This is intriguing since it has been shown that CH causes up regulation of HIF-1 with subsequent increase in EPO production and EPO receptor expression [18]. One the mechanisms of EPO-mediated increases in neurogenesis, is thought to be by inducing brain derived neurotropic factor (BDNF) in the hippocampus [19]. Previous studies in adult models of CH show significantly decreased hippocampal levels of BDNF [20]. Since one of the mechanisms of increased neurogenesis by EPO is through BDNF, the presence of increased endogenous EPO seen in adult CH may be insufficient to rescue the neurogenesis during hippocampal development. This protective effect of EPO on hippocampal neurogenesis in CH could be further supported by hippocampal based behavior studies in the future.

Studies have shown that neonatal hypoxia-ischemia cause disturbances in OPC maturation [9] another important progenitor cell lineage critical for brain development. Similar observations in our novel pediatric CH model confirm that CH, even at later stages of development can still decreases the OPC pool with subsequent loss of myelination in conjunction with decreased neurogenesis. Treatment with EPO increases the number of OPC as seen in other models of injury such as hypoxia-ischemia, however, with the same dose we only see a trend towards improvement in myelination. We do know that maturation of OPCs to mature myelin is a complex process with the expression of multiple differentiation genes [21]. Therefore EPO alone may not be able to rescue the loss of myelin in our pediatric mouse model of CH, particularly as most of the protective effect of EPO on myelination at the dose used in our study occurs in neonatal mice and in cell culture models [13]. Furthermore, the blood brain barrier is much more developed in the age group in our study compared to the neonatal mice, which could contribute to low-dose EPO efficacy during earlier stages of neural development. This would suggest that a dose–response curve should be established in future studies in the use of EPO therapy after pediatric injury.

This is the first evaluation of EPO in a non neonatal pediatric [13] model of hypoxia-induced brain injury. EPO therapy promoted neurogenesis and OPC development when given during the time of prolonged hypoxia exposure, increasing early, late and actively dividing stem cells in the dentate gyrus. We did not see any increased mortality or any obvious neurological deficits in pediatric EPO-treated mice. This is important in the evaluation as a clinical therapy, as the use of EPO in adult models of brain injury increased the incidence of stroke [22]. The safety in a pediatric population was confirmed since EPO did not have a negative effect in normoxic control mice. This is due to the fact that the upregulation...
of EPO-R is under the control of HIF-1 and therefore only induced under hypoxic conditions rendering EPO therapy in uninjured mice ineffective.

5. Conclusions

As improvements in technology lead to increased survival in pediatric CH states, neurologic morbidity as a consequence of CH will increase in incidence. Numerous neuroprotective therapies have been studied, but there is currently no effective pharmacologic therapy for neuroprotection in pediatric CH. In our study, we were able to establish increased neurogenesis and preservation of OPCs for pediatric mice given EPO under CH conditions. As EPO is an FDA-approved therapy with ongoing clinical trials for neonatal brain injury, further studies are warranted to investigate the potential use of EPO as a neuroprotective agent in pediatric CH.

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References
