Int. J. Devl Neuroscience 43 (2015) 35–42

Contents lists available at ScienceDirect

International Journal of Developmental Neuroscience

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

Hypoxic preconditioning can reduce injury-induced inflammatory processes in the neonatal rat brain

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ARTICLE INFO

Article history:
Received 18 February 2015
Received in revised form 26 March 2015
Accepted 26 March 2015
Available online 28 March 2015

Keywords:
Neonatal
Hypoxia-ischemia
Hypoxic preconditioning
Inflammation
Neuroprotection

ABSTRACT

Inflammation plays an important role in the pathophysiology of neonatal hypoxic–ischemic (HI) brain injury. Studies have shown that hypoxic preconditioning (HP) can ameliorate brain damage, but its effects on inflammation remain unknown. Postnatal day 6 (P6), Sprague–Dawley rats were divided into normoxia and hypoxia (8% oxygen, 3 h) groups. On P7, some pups underwent a right carotid artery occlusion followed by hypoxia (8% oxygen, 3 h) while under 1.5% isofluorane anesthesia and the remaining pups underwent sham surgery without occlusion. Animals were sacrificed 5 days later and fixed tissue was used to examine changes in neurons, astrocytes, and microglia in the cortex. Fresh tissue was collected to determine cortical levels of proinflammatory cytokines using ELISA. There was a significant loss in the number of NeuN positive cells in the cortex following HI injury, which was improved when HP was given prior to HI. There was an increase in cortical area of astrocyte staining after HI injury compared to control. HP before HI was able to reduce area of GFAP staining back to control levels. HI caused a large increase in the number of activated microglia compared to control and HP was able to significantly reduce this, although not back to control levels. HP alone increased microglial activation. Interleukin-1β levels were increased in the cortex 5 days after HI, but HP was not able to significantly reduce this change. The neuroprotective effects of HP appear to be mediated by affecting cellular inflammatory processes in the brain following HI injury.

1. Introduction

Hypoxic–ischemic (HI) brain injury during birth or *in utero* remains a major cause of morbidity and mortality in newborns resulting from impaired blood and oxygen delivery to the brain (Berger et al., 2002). This type of brain injury affects approximately 4–6 per 1000 live born infants (Badawi et al., 1998; Vannucci and Hagberg, 2004), of which 20% die, whereas, 25% are left with long-term neurological disabilities (Glass et al., 2011; Lynch et al., 2012). Hypothermia is used clinically to improve some outcomes following HI, but is not effective in all infants (Jacobs et al., 2013).

Following HI injury, a robust glial response is initiated in the brain involving astrocytes and microglia. Astrocytes have important roles in the developing brain including synaptic maintenance (Christopherson et al., 2005), metabolism, glutamate uptake and clearance, and blood–brain barrier formation (Sofroniew and Vinters, 2010). In the neonatal rat brain, following an injury, astrocytes proliferate and undergo hypertrophy, invading the injury site to form a barrier between healthy and damaged tissue (Sizonenko et al., 2008). These processes aid the surrounding brain tissue in multiple ways including clearing up excessive glutamate and maintaining ionic gradients and metabolic processes. In contrast, activated astrocytes are also able to release proinflammatory cytokines which are likely to worsen the injury (Lee et al., 1993).

Microglia, the resident immune surveillance cells in the brain play a role in neurogenesis and perform synaptic pruning in the developing brain (Harry and Kraft, 2012). Neonatal HI injury causes activation of microglia (Benjelloun et al., 1999), which infiltrate the injury site and clear away cellular debris. Microglial activation and aggregation has been observed in human infants with HI brain

Abbreviations: BSA, bovine serum albumin; DAB, diaminobenzidine; EDTA, ethylenediamminetetraacetic acid; ELISA, enzyme linked immunosorbent assay; EPO, erythropoietin; GFAP, glial fibrillary acidic protein; HI, hypoxia-ischemia; HIF, hypoxia inducible factor; HP + HI, hypoxic preconditioning and hypoxia-ischemia; HP, hypoxic preconditioning; IBA-1, ionized calcium-binding adapter molecule 1; IL, interleukin; NeuN, neuronal nuclei; P, postnatal day; PFA, paraformaldehyde; PBS, phosphate buffered saline; RT, room temperature; SD, Sprague–Dawley; SEM, standard error of mean; Strep-HRP, strepdavidin horseradish peroxidase; TGF, transforming growth factor; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.

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http://dx.doi.org/10.1016/j.ijdevneu.2015.03.010
0736-5748/

damage (Del Bigio and Becker, 1994). Stimulation of neuroinflammatory processes following activation of microglia can exacerbate neurological damage following HI injury (Tang et al., 2014), where cytokine production can lead to apoptosis. Cytokines are the main mediators the inflammatory response in the brain. Infants with HI-induced brain damage have higher serum and cerebrospinal fluid levels of proinflammatory cytokines, interleukin-1β (IL-1β), and tumor necrosis factor-α (TNF-α) compared to control. Similarly, neonatal rats with HI injury have an increased mRNA and protein levels of IL-1β and TNF-α (Szaflarski et al., 1995; Hagberg et al., 1996; Hu et al., 2005).

Over the past two decades, hypoxic preconditioning (HP) has been used as a neuroprotective tool to study mechanisms that underlie endogenous brain repair pathways. HP involves exposing the brain to a mild level of hypoxia that can alter signalling pathways, which can protect against a more severe injury (Park et al., 2011). The neuroprotective effects of HP are mediated *via* the transcription factor hypoxia-inducible factor-1 (HIF-1) (Bergeron et al., 2000; Sharp et al., 2001) and various target genes, which initiate angiogenesis, erythropoiesis, glucose store mobilization and therefore, cell survival. Recent studies have shown the protective effects of hypoxia to be mediated by HIF-1α, where knockout mice were not protected by HP (Sheldon et al., 2014), and pharmacological blockade of HIF-1 was able to prevent hypoxia-induced microglial activation (Huang et al., 2014).

Studies have shown that HP prior to HI can suppress levels of various cytokines and chemokines in the neonatal rat brain (Yin et al., 2007). Similarly, remote ischemic preconditioning (Albrecht et al., 2013) and intermittent hypoxia in patients with sleep apnoea (Ryan et al., 2005) can reduce serum levels of IL-1β and TNF-α, among other inflammatory markers in the blood. Here, we have investigated the effects of HP on inflammatory cells, astrocytes, and microglia and levels of proinflammatory cytokines IL-1β and TNF-α in neonatal rat model of HI injury.

2. Methods

2.1. Animals

All procedures were performed in accordance with the National Health and Medical Research Council ethical code, along with approval from the University of New South Wales Animal Care and Ethics Committee. Lactating female Sprague–Dawley rats and their pups were obtained from the Animal Resources Center, Perth, Australia. Animals were housed at constant room temperature (RT) of 23 °C with a 12 h light and dark cycle. The brain tissue used for immunohistochemistry in this study was generated in a previous study where we examined white matter injury (Suryana and Jones, 2014).

2.2. Hypoxic preconditioning

Postnatal day (P) 6 pups were divided into a normoxic group ($n = 36$) and HP group ($n = 34$). Pups in the HP group were exposed to 8% oxygen balanced with 92% nitrogen by placing them in a large plexiglass hypoxic chamber (BioSpherix, Redfield, NY) for 3 h while the normoxic group were kept in room air for 3 h. During this time, all pups were kept on a heating mat to prevent hypothermia.

2.3. HI surgical procedure

On P7, pups in both groups were further subdivided into injury and sham treatment making four treatment groups in total for this study (HP, $n = 13$; HP + HI, $n = 21$; control, $n = 12$; and HI, $n = 25$). Pups underwent anesthesia with 1.5% isofluorane through inhalation and HI injury was produced by permanently occluding the right common carotid artery (Rice et al., 1981) using an electrocautery device (World Precision Instruments, Sarasota, FL, USA). Control and HP pups underwent sham surgery with no occlusion. After 1.5 h of recovery with the mother, pups were placed in the hypoxic chamber (8% O_2, balanced with 92% N_2) for 3 h while the sham treated pups were kept in room air. All pups were kept on a heating mat to prevent hypothermia.

2.4. Immunohistochemical staining for cellular markers

Brain tissue was collected on P13. Pups were euthanized with Lethabarb (100 mg/kg; i.p.) and transcardially perfused with phosphate buffered saline (PBS), followed by 4% paraformaldehyde (PFA). Brains were removed, postfixed for 4 h with 4% PFA and stored in 30% sucrose. Brains were frozen prior to sectioning (60 μm) on a cryostat and sections stored at −20 °C in cryoprotectant until use. Free floating coronal sections from ~Bregma 3.70 mm to −4.80 mm, 60 μm thick (~1440 μm apart) from each animal (3–4 sections) were immunostained for neuronal nuclei (NeuN), glial fibrilliary acidic protein (GFAP), and ionized calcium binding adapter protein-1 (Iba1). Briefly, sections were washed in PBS and antigen retrieval was performed using sodium citrate buffer (10 mM, pH 7.5) at 70 °C for 10 min. Blocking solution (5% non-fat skim milk powder, 2% horse serum for NeuN and GFAP, and 2% rabbit serum for Iba1, 0.1% BSA, 0.3% Triton X-100 in PBS) was used for non-specific blocking. Sections were incubated overnight at 4 °C with diluted mouse anti-NeuN monoclonal antibody (Millipore, CA), mouse monoclonal anti-GFAP (Millipore, CA), or goat polyclonal anti-Iba1 antibody (Abcam, Waterloo, NSW) [made in primary antibody solution in 1:1000 concentration; 1% serum (horse serum for NeuN and GFAP and rabbit serum for Iba1), 0.1% BSA, 0.3% Triton X-100].

On the following day, sections were incubated in 0.1% hydrogen peroxide (in PBS) to block endogenous peroxidases, biotinylated secondary antibody (mouse for NeuN and GFAP and goat for Iba1; made in antibody solution; concentration 1:200; Vector laboratories; 2 h), strepdavidin–horseradish peroxidase (1:500 in PBS; Vector laboratories; 2 h) and stained with diaminobenzidine (Vector laboratories; 2.5 min). In between each step, sections were washed with static-free solution (0.05% BSA, 0.1% Tween-20 in PBS, 5 min) and PBS (2 × 5min). The sections were mounted on glass slides using 0.3% gelatine. Sections were air-dried, dehydrated using 70%, 95%, and 100% alcohol concentrations, rinsed in histolene (Grale Scientific, AUS) and coverslipped with DPX mounting medium (BDH, England).

2.5. Quantification of cellular markers

Cell counting of NeuN positive cells was performed at 40× magnification to estimate the number of surviving neurons throughout the ipsilateral cortex using stereology (Oorschot, 2011). Neurons from four serial sections 1440 μm apart were estimated using the optical fractionator probe in StereoInvestigator V9 program (MBF bioscience, USA) along with brightfield microscopy. The boundary of the ipsilateral cortex was marked in each section using a free hand marking tool in the program. The entire cortex was then divided into 800 μm^2 grid in which neurons were counted in 100 μm^2 area within 3 μm thickness. The following equation was employed to estimate neuronal count per section (West et al., 1991).

$$N = \sum Q^{-} \times \frac{1}{\text{tsf}} \times \frac{1}{\text{asf}} \times \frac{1}{\text{ssf}}$$

where (N = estimate of the total number of NeuN-positive cells; Q^{-} = sum of NeuN-positive cells counted in the counting frame; tsf = thickness sampling fraction = dissector height ÷ section thickness; ssf = section sampling fraction = number of sections