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Alterations in inter-alpha inhibitor protein expression after hypoxic-ischemic brain injury in neonatal rats

Clémence Disdier[a], Jiyong Zhang[a], Yuki Fukunaga[b], Yow-Pin Lim[c], Joseph Qiu[c], Andre Santoso[c], Barbara S. Stonestreet[a,*]

[a] Department of Pediatrics, Women & Infants Hospital of Rhode Island, The Alpert Medical School of Brown University, Providence, RI 02905, USA
[b] Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, Okayama University, Okayama 7008558, Japan
[c] ProThera Biologics, Inc., Providence, RI 02903, USA

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ABSTRACT

Hypoxic-ischemic (HI) brain injury is frequently associated with premature and/or full-term birth-related complications that reflect widespread damage to cerebral cortical structures. Inflammation has been implicated in the long-term evolution and severity of HI brain injury. Inter-Alpha Inhibitor Proteins (IAIPs) are immune modulator proteins that are reduced in systemic neonatal inflammatory states. We have shown that endogenous IAIPs are present in neurons, astrocytes and microglia and that exogenous treatment with human plasma purified IAIPs decreases neuronal injury and improves behavioral outcomes in neonatal rats with HI brain injury. In addition, we have shown that endogenous IAIPs are reduced in the brain of the ovine fetus shortly after ischemic injury. However, the effect of HI on changes in circulating and endogenous brain IAIPs has not been examined in neonatal rats. In the current study, we examined changes in endogenous IAIPs in the systemic circulation and brain of neonatal rats after exposure to HI brain injury. Postnatal day 7 rats were exposed to right carotid artery ligation and 8% oxygen for 2 h. Sera were obtained immediately, 3, 12, 24, and 48 h and brains 3 and 24 h after HI. IAIPs levels were determined by a competitive enzyme-linked immunosorbent assay (ELISA) in sera and by Western immunoblots in cerebral cortices. Serum IAIPs were decreased 3 h after HI and remained lower than in non-ischemic rats up to 7 days after HI. IAIP expression increased in the ipsilateral cerebral cortices 24 h after HI brain injury and in the hypoxic contralateral cortices. However, 3 h after hypoxia alone the 250 kDa IAIP moiety was reduced in the contralateral cortices. We speculate that changes in endogenous IAIPs levels in blood and brain represent constituents of endogenous anti-inflammatory neuroprotective mechanism(s) after HI in neonatal rats.

1. Introduction

Hypoxic-Ischemic (HI) brain injury is a result of decreased blood flow to the brain combined with lower-than-normal oxygen concentrations in arterial blood (Dixon et al., 2015; Mehta et al., 2007). HI related events in premature and full-term infants increase mortality and result in long-term neurological deficits including cerebral palsy, epilepsy and seizure disorders, severe learning and mental impairment, cognitive, motor and behavioral developmental problems (Conklin et al., 2008; Fatemi et al., 2009; Kharoshankaya et al., 2016; Pappas et al., 2015). The only currently available strategy to attenuate brain injury in newborns is therapeutic hypothermia (Gluckman et al., 2006; Jacobs et al., 2013; Natarajan et al., 2016; Shankaran, 2012). This therapy is only approved for use in full-term newborns with hypoxic ischemic encephalopathy (HIE) and, unfortunately, is only partially neuroprotective (Gluckman et al., 2006; Jacobs et al., 2013; Natarajan et al., 2016; Shankaran, 2012).

Post-ischemic neuroinflammation is a key pathophysiological factor in the evolution of HI-related brain injury (Ferriero, 2004; Hagberg et al., 2015; Lai et al., 2017; Riljak et al., 2016; Rocha-Ferreira and Hristova, 2016). The first phase of HI injury lasts minutes to hours after the initial insult and is marked by oxidative stress and depletion of energy stores. The second phase of HI injury occurs from hours to days after the insult and is characterized by an intense neuroinflammatory response. This neuroinflammatory response is associated with the release of several pro-inflammatory cytokines/chemokines, recruitment of proteases, activation of resident immune cells (*e.g.* microglia) and infiltration of circulating immune cells (*e.g.* circulating monocytes)

* Corresponding author at: The Alpert Medical School of Brown University, Department of Pediatrics, Women & Infants Hospital of Rhode Island, 101 Dudley Street, Providence, RI, 02905, USA.
E-mail address: bstonestreet@wihri.org (B.S. Stonestreet).

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(Jellema et al., 2013; Liu and McCullough, 2013), which exacerbates brain injury and contributes to later adverse outcomes. Consequently, attenuation of these inflammatory processes could represent a promising strategy to reduce and/or prevent brain damage after HI injury in neonates. However, the inflammatory response that initially is harmful may be beneficial later because inflammation also contributes to many repair processes. Therefore, the timing of initiation of anti-inflammatory therapeutics is critical to the final outcome of brain injury.

Inter-alpha inhibitor proteins (IAIPs) are a family of endogenous serine protease inhibitors found in blood and numerous fetal, neonatal and adult tissues including the brain (Chen et al., 2016; Salier et al., 1996; Spasova et al., 2014; Takano et al., 1999). Two moieties are found in mammalian plasma: Inter-alpha Inhibitor (*IαI*) composed of two heavy chains (H1 and H2) and a single light chain also called bikunin, and a Pre-alpha Inhibitor (*PαI*), composed of one heavy (H3) and one light chain. IAIPs have already been shown to have systemic anti-inflammatory properties by inhibiting destructive serine proteases, blocking complement activation, and pro-inflammatory cytokines production as well as by promoting the production of anti-inflammatory cytokines (Fries and Blom, 2000; Fries and Kaczmarczyk, 2003; Garantziotis et al., 2007; Okroj et al., 2012; Singh et al., 2010). IAIPs have been shown to be reduced in the plasma of premature infants who have systemic inflammatory disorders including sepsis and necrotizing enterocolitis (Baek et al., 2003; Chaaban et al., 2010; Chaaban et al., 2009). These findings suggest that inflammatory disorders adversely affect systemic endogenous IAIPs levels in premature neonates. However, there is limited information regarding the effects of HI brain injury on systemic and brain levels of endogenous IAIPs in neonates.

Endogenous IAIPs are expressed in relatively high amounts during development in rodent and ovine brain and are detected in neurons, microglia, astrocytes and oligodendrocytes, and in multiple brain regions including cerebral cortex and hippocampus in both neonatal rats and mice (Chen et al., 2016; Spasova et al., 2014). The ubiquitous presence of endogenous IAIPs in numerous types of brain cells and brain regions in the central nervous system (CNS) of rodents suggests that endogenous IAIPs represent an important, but previously unrecognized constituent of the normal brain composition, and most likely function (Chen et al., 2016; Spasova et al., 2014). In addition, we have previously shown that IAIP expression is reduced shortly after ischemia in the cerebral cortex and cerebellum of fetal sheep and returns toward non-ischemic levels between 24 and 48 h after an ischemic insult (Spasova et al., 2016). These findings suggest that ischemia is associated with increased IAIP utilization and/or decreased production in the CNS (Spasova et al., 2016). Nonetheless, brain ischemia in the fetal sheep did not result in alterations in plasma concentrations of IAIPs (Spasova et al., 2016). Therefore, IAIPs represent endogenous anti-inflammatory molecules that could be regulated in the brain during injury related events.

Recent work has also shown that exogenous treatment with IAIPs in neonates exposed to HI reduces neuronal cell death, improves neuronal plasticity, ameliorates complex auditory processing deficits, cognitive function, and behavioral outcomes (Gaudet et al., 2016; Threlkeld et al., 2014, 2017). Administration of urinary bikunin, the light chain of IAIPs demonstrated neuroprotective properties by reducing pro-inflammatory mediators and resulting in milder ischemia related brain injury in young piglets (Wang et al., 2013). The results of the above studies support the contention that IAIPs are endogenously present in brain, levels can be affected by HI injury, and that exogenous treatment with IAIPs potentially have promising neuroprotective properties after neonatal HI. The importance of inflammation in HI related brain damage and the immunomodulatory properties of IAIPs suggest that these proteins could be promising therapeutics to attenuate brain injury after HI in neonates.

Given the above considerations, the objective of the current study was to extend our previous findings to examine potential alterations in endogenous IAIPs in the systemic circulation and brain of neonatal rats after exposure to HI brain injury.

2. Methods

This study was conducted with the approval by the Institutional Animal Care and Use Committees of the Alpert Medical School of Brown University and Women & Infants Hospital of Rhode Island and in accordance with the National Institutes of Health Guidelines for the use of experimental animals.

2.1. Animal preparation, study groups, and experimental design

Subjects were Wistar rats born from time-mated dams obtained from Charles River Laboratory (Wilmington, Maine, USA). Post-natal (P) day 7 rats were randomly assigned to sham control or HI groups. The Rice-Vannucci method was used to induce the HI injury (Rice et al., 1981). Briefly, each animal was anesthetized with 3–4% isoflurane and anesthesia maintained with 1% isoflurane. Total absence of leg withdrawal reflexes was verified before the onset of surgery. A midline ventral incision was made in the neck. The right common carotid artery (RCCA) was located and ligated. Sham subjects were exposed to the same procedure except the RCCA was not ligated. Body temperature was maintained at 36 °C during surgery with an isothermic heating pad (Marks et al., 2010; Mishima et al., 2004; Reinboth et al., 2016; Silveira and Procianoy, 2015). The pups were returned to their dams for 1.5–3 h for feeding and recovery from surgery before exposure to hypoxia (8% oxygen). Subjects exposed to HI were placed in a hypoxia chamber with 8% humidified oxygen and balanced nitrogen for 2 h with a constant temperature of 36 °C. Sham subjects were exposed to room air for 2 h. The pups were sham treated (n = 8 females/10 males) or remained with their dams until 3 h (n = 2 females/3 males), 6 h (n = 3 females/3 males), 12 h (n = 5 females/8 males), 24 h (n = 1 female/4 males), 48 h (n = 3 females/2 males), and 7 days (n = 1 female/3 males) after HI when they were euthanized. Three hundred to 500 μL of whole blood were collected without the use of anticoagulant from the left ventricle and centrifuged immediately at 5000 rpm at room temperature. The clots were removed and the supernatant collected in a fresh tube and placed on ice for 2 h. Any additional clots that formed were also removed and the supernatant saved at −80 °C until analysis. The samples were again centrifuged before analysis and the supernatant were used for ELISA.

Brain tissue was obtained from the sham, and HI animals at 3 h and 24 h (n = 8 for right cortices and n = 6–8 for left cortices in each group) after exposure to HI. Brains were dissected to isolate the right HI or left non-ischemic hypoxic cerebral cortices in the sham and HI groups. The right cerebral cortex represented the hypoxic-ischemic damaged brain tissue. The left cerebral cortex represented the tissue exposed to hypoxia, but not ischemia. Brain tissues for this study were residual samples from earlier projects, and consequently, right and left cortices were not available from the same animals and could not be compared.

2.2. Competitive ELISA to measure IAIPs concentrations in rat sera

Serum concentrations of IAIPs in sham and HI rats were measured by a competitive ELISA using a polyclonal antibody raised against rat IAIPs (R-21 pAb). The R-21 pAb was generated by immunization of rabbits with rat serum derived IAIPs that cross-react with mouse and human IAIPs and detect both the 250 kDa *IaI* and 125 kDa *PaI* proteins by Western immunoblot analysis. The R-21 pAb was purified by affinity chromatography using rProtein-A column (Tosoh Bioscience, Tokyo, Japan) and purified IgG was conjugated with biotin using an EZ-Link NHS-LC-Biotin kit (Thermo Scientific, Wilmington, DE, USA). Rat IAIPs that were used to establish the standard curve for quantitative analysis were extracted and purified as previously described for human IAIPs