Int. J. Devl Neuroscience 48 (2016) 58–70

Contents lists available at ScienceDirect

International Journal of Developmental Neuroscience

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

Endoplasmic reticulum pathology and stress response in neurons precede programmed necrosis after neonatal hypoxia-ischemia

Raul Chavez-Valdez[a,*], Debbie L. Flock[a], Lee J. Martin[b], Frances J. Northington[a,*]

[a] Department of Pediatrics, Neonatal Research Laboratory, Johns Hopkins University School of Medicine, 600 N. Wolfe Street, CMSC 6-104, Baltimore, MD 21287, USA

[b] Departments of Pathology and Neuroscience, Johns Hopkins University School of Medicine, 720 Rutland Ave. Ross Research Building, Room 558, Baltimore, MD 21205, USA

ARTICLE INFO

Article history:
Received 26 October 2015
Received in revised form
24 November 2015
Accepted 24 November 2015
Available online 28 November 2015

Keywords:
Endoplasmic reticulum stress
Programmed necrosis
Unfolded protein response
Neonatal hypoxia-ischemia
Cell death
Cytoplasmic shedding
Macrozeiosis

ABSTRACT

The endoplasmic reticulum (ER) is tasked, among many other functions, with preventing excitotoxicity from killing neurons following neonatal hypoxia-ischemia (HI). With the search for delayed therapies to treat neonatal HI, the study of delayed ER responses becomes relevant. We hypothesized that ER stress is a prominent feature of delayed neuronal death via programmed necrosis after neonatal HI. Since necrostatin-1 (Nec-1), an inhibitor of programmed necrosis, provides delayed neuroprotection against neonatal HI in male mice, Nec-1 is an ideal tool to study delayed ER responses. C57B6 male mice were exposed to right carotid ligation followed by exposure to $FiO_2 = 0.08$ for 45 min at p7. Mice were treated with vehicle or Nec-1 (0.1 μl of 8 μmol) intracerebroventricularly with age-matched littermates as controls. Biochemistry assays at 3 and 24 h and electron microscopy (EM) and immunohistochemistry at 96 h after HI were performed. EM showed ER dilation and mitochondrial swelling as apparent early changes in neurons. With advanced neurodegeneration, large cytoplasmic fragments containing dilated ER "shed" into the surrounding neuropil and calreticulin immunoreactivity was lost concurrent with nuclear features suggestive of programmed necrosis. Nec-1 attenuated biochemical markers of ER stress after neonatal HI, including PERK and eIF2α phosphorylation, and unconventional XBP-1 splicing, consistent with the mitigation of later ER pathology. ER pathology may be an indicator of severity of neuronal injury and potential for recovery characterized by cytoplasmic shedding, distinct from apoptotic blebbing, that we term neuronal macrozeiosis. Therapies to attenuate ER stress applied at delayed stages may rescue stressed neurons after neonatal HI.

© 2015 Elsevier Ltd. All rights reserved.

1. Introduction

Aberrant Ca^{2+} regulation and oxidative damage (Ferriero, 2004; Puka-Sundvall et al., 2000) are central to the basic biology of brain injury following neonatal hypoxia-ischemia (HI). The endoplasmic reticulum (ER) is tasked with preventing these insults from killing neurons (Paschen, 2003) in vivo and in cell culture. The ER responds to HI with morphologic and biochemical changes designed to contain and block propagation of these deleterious events. Initiation of the unfolded protein response (UPR) in response to neonatal HI is documented (Badiola et al., 2011; Carloni et al., 2014a) and UPR mitigation is associated with neuroprotection in multiple neonatal brain injury models with a variety of therapies (Carloni et al., 2014a,b; Zhu et al., 2014).

Studies of ER stress in neurodegenerative conditions suggests a role in failed neuroprotection or neuronal maintenance on a more chronic basis (Paschen and Mengesdorf, 2005). ER stress and autophagy pathways can cooperate as well (Sheng et al., 2012). Morphologic changes in ER structure are linked to stimuli known to cause neurodegeneration. Enlargement of the intraluminal ER capacity is considered a survival mechanism designed to allow more time for handling of misfolded proteins in culture (Bernales et al., 2006) and greater sequestration of Ca^{2+}. ER stress signaling

Abbreviations: ATF, activation transcription factor; CHOP, CCAAT/-enhancer-binding protein homologous protein; EIF, eukaryotic initiation factor; EM, electron microscopy; ER, endoplasmic reticulum; GADD, growth arrest and DNA damage-inducible protein; GRP78, 78 kd glucose regulated protein (also known as BiP and HSP5a); HI, hypoxia-ischemia; IQR, interquartile range; IRE, inositol-requiring enzyme; Nec-1, necrostatin-1; p, postnatal day; PERK, protein kinase RNA-like endoplasmic reticulum kinase; UPR, unfolded protein response; XBP, X-box protein.

* Corresponding authors at: Department of Pediatrics, Division of Neonatology, Johns Hopkins Hospital, 600 N. Wolfe Street, CMSC 6-104, Baltimore, MD 21287, USA. Fax: +1 410 614 8388.

E-mail addresses: rchavez2@jhmi.edu (R. Chavez-Valdez), dflock1@jhmi.edu (D.L. Flock), martinl@jhmi.edu (L.J. Martin), frances@jhmi.edu (F.J. Northington).

http://dx.doi.org/10.1016/j.ijdevneu.2015.11.007
0736-5748/© 2015 Elsevier Ltd. All rights reserved.

is acutely activated in models of neonatal brain injury. Rough ER containing intraluminal Ca^{2+} precipitates are present in immature rat brain after HI (Puka-Sundvall et al., 2000); however, no investigation into ER stress beyond the acute injury period in neonatal brain injury has been undertaken.

We have shown that a delayed phase of neurodegeneration is a major component of neonatal brain injury after HI (Northington et al., 2001). Hence, we hypothesized that ER stress is a prominent feature of this delayed neuronal injury and that it may be part of the programmed necrosis form of neurodegeneration that we have described associated with neonatal HI (Chavez-Valdez et al., 2012a,b; Northington et al., 2011). Importantly, necrostatin-1 (Nec-1), an allosteric inhibitor of receptor interacting protein kinase 1, blocks mitochondrial failure and oxidative injury to proteins during the acute phase after neonatal HI in mouse (Chavez-Valdez et al., 2012a; Northington et al., 2011); yet, its neuroprotective effect is not evident until 96 h after injury (Northington et al., 2011). With the search for therapies to apply beyond the acute phase of injury, investigation of delayed ER stress is highly pertinent in neonatal brain injury research and Nec-1 is an ideal therapeutic tool to that end.

In this study we sought to determine (i) whether ER stress in neurons has a prominent presence after the acute phase of injury as witnessed by ultrastructural assessment, (ii) how ultrastructural pathology may relate to the progression toward neurodegeneration, and (iii) the efficacy of Nec-1 to mitigate early biochemical indicators of ER stress and delayed ER pathology.

2. Methods and methods

2.1. Animals

Neonatal male mice were used. Experiments were performed with approval by the Institutional Animal Care and Use Committee at Johns Hopkins University School of Medicine and they were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (8th edition, NIH Publications) revised in 2011 and EU Directive 2010/63/EU for animal experiments. A total of 72 male mice were used for experiments. All efforts were made to minimize the number of mice used and their suffering. Male mice were chosen for the present study because in our hands, they sustain a more consistent injury after HI and have demonstrated a more consistent therapeutic response to treatment with Nec-1 at the same dose used for the current experiments (Northington et al., 2011).

2.2. Rice-Vannucci model of neonatal HI brain injury

HI was induced in male C57B6 mice at postnatal day (p) 7 using a modification of the Rice-Vannucci procedure for mice (Ditelberg et al., 1996). Mice were exposed to a second brief period of anesthesia with isofluorane followed by an intracerebroventricular (ICV) injection of 0.1 μl of 8 μmol Nec-1 (Calbiochem-EMD Chemicals Group, Gibbstown, NJ), or vehicle, methyl-β-cyclodextrin (Sigma, St. Louis, MO, USA) 15 min after the end of 45 min of hypoxic exposure (FiO_2 = 0.08). The dose of Nec-1 used for the current set of experiments has been proven to attenuate early biochemical changes and prevent delayed histological injury in forebrain of neonatal male mice exposed to experimental HI (Chavez-Valdez et al., 2012a; Northington et al., 2011). Pups were killed at 3 h and 24 h after HI (n = 6–8 pups/time/treatment) for biochemical analysis. Naive controls were age-matched littermates not exposed to HI or treatments. Mice were killed with an exposure to 20% (v/v) mixture of isoflurane in propylene glycol via one drop exposure method (Markovic and Murasko, 1993) and then decapitated. Brain was micro-dissected separating the hemispheres at the level of longitudinal cerebral fissure, removing the cerebellum at the level of transverse fissure, and isolating tissue above and lateral to the fornix to include hippocampus, striatum and cortex (forebrain) as previously described (Chavez-Valdez et al., 2014). Tissues were rapidly frozen (−80 °C) for protein and qRT-PCR analysis (n = 4 pups/treatment/time/experiment). A separate group of mice were used for electron microscopy (EM) and immunohistochemistry IHC) as described in Section 2.3.

2.3. Electron microscopy and immunohistochemistry tissue preparation

For EM and IHC, animals were killed with an overdose of isoflurane as above, and exsanguinated with cold 0.1 M PBS (pH 7.4) via intra-cardiac perfusion (n = 12, 4 pups/treatment). Brains were perfusion fixed with either 2% gluteraldehyde/2% paraformaldehyde for EM, or 4% paraformaldehyde/0.1 M PBS for 30 min at 4 ml/min for IHC. Tissue was prepared for EM at the Cell Biology Imaging Facility, Johns Hopkins University. Briefly, tissue pieces from ipsilateral sensory motor cortex (3 mm^3) were fixed in 2% paraformaldehyde, 2% glutaraldehyde, 0.1 M sodium cacodylate, 3 mM CaCl2, at pH 7.4 overnight at 4 °C. Following buffer rinse, samples were post-fixed in 1% osmium tetroxide, 0.1 M sodium cacodylate, 3 mM CaCl2 for 2 h on ice in the dark. After a brief dH_2O rinse, samples were incubated in 2% uranyl acetate for 1 h at room temperature, in the dark. Following en-bloc staining tissue samples were dehydrated through a graded series of ethanol to 100%, transferred through propylene oxide and embedded in Pelco Eponate 12 (Ted Pella. Inc., Redding, CA) and cured at 60 °C for two days. Sections were cut on a Reichert Ultracut E with a 35° angle (compression free) Diatome Diamond knife. Thin sections (80 nm) were collected on formvar coated 1 × 2 mm copper slot grids and stained with uranyl acetate followed by lead citrate. Grids were viewed on a Hitachi 7600 TEM operating at 80 kV and digital images captured with an AMT XR-50 (5 megapixel) CCD camera.

For IHC tissues were cryoprotected with graded immersion in 15% and then 30% sucrose in PBS until the tissue sank, then frozen and stored at −80°C until cut at 50 μm on a freezing microtome (Northington et al., 1996). Floating IHC for calreticulin was performed as previously described (Northington et al., 1996) with 1:500 dilution of whole rabbit antisera anti-calreticulin antibody (Novus Biologicals USA, Littleton, CO). Goat anti-rabbit antibody (1:200) was used as the secondary antibody and 3,3′-diaminobenzidine (DAB) as the chromagen (Northington et al., 1996). Tissues were counterstained with cresyl violet after developing in DAB.

Cortical neurons were assessed by EM and IHC to determine the intermediate stages of ER pathology in those injured neurons that survived neonatal HI at delayed stages. Morphological ER features in cortical neurons were most relevant to study given that forebrain tissue, which is mostly cortical tissue, was used for the biochemical assays. Because of the severity of hippocampal injury soon after neonatal HI and the smaller contribution of hippocampus in the forebrain homogenates, hippocampal neurons were considered less well suited for evaluating delayed ER pathology and cell death.

2.4. Gene expression by real time qRT-PCR

Total RNA was extracted from forebrain harvested from mice exposed to neonatal HI and treatments as described above (n = 4 pups/treatment/time). PureLink™ RNA mini kit purification system (Invitrogen, Carlsbad, CA) was used according to manufacturer specifications. Approximately 1 μg of total RNA was used for generation of complementary DNA (cDNA) using iScript cDNA synthesis

Download English Version:

https://daneshyari.com/en/article/2785633

Download Persian Version:

https://daneshyari.com/article/2785633

Daneshyari.com