SEX-DEPENDENT MITOPHAGY AND NEURONAL DEATH FOLLOWING RAT NEONATAL HYPOXIA–ISCHEMIA

T. G. DEMAREST, [a,d] E. L. WAITE, [b] T. KRISTIAN, [a,f]
A. C. PUCHE, [d,e] J. WADDELL, [c] M. C. MCKENNA [c,d] AND
G. FISKUM [a,d]*

[a] *Department of Anesthesiology and the Center for Shock, Trauma, and Anesthesiology Research (S.T.A.R.), University of Maryland School of Medicine, Baltimore, MD 21201, USA*

[b] *Department of Chemistry and Biochemistry, University of Maryland, College Park, MD 21201, USA*

[c] *Department of Pediatrics, University of Maryland School of Medicine, Baltimore, MD 21201, USA*

[d] *Program in Neuroscience, University of Maryland School of Medicine, Baltimore, MD 21201, USA*

[e] *Department of Anatomy and Neurobiology, University of Maryland School of Medicine, Baltimore, MD 21201, USA*

[f] *Veterans Affairs Medical Center, University of Maryland School of Medicine, Baltimore, MD 21201, USA*

Abstract—Males are more susceptible than females to long-term cognitive deficits following neonatal hypoxic-ischemic encephalopathy (HIE). Mitochondrial dysfunction is implicated in the pathophysiology of cerebral hypoxia–ischemia (HI), but the influence of sex on mitochondrial quality control (MQC) after HI is unknown. Therefore, we tested the hypothesis that mitophagy is sexually dimorphic and neuroprotective 20–24 h following the Rice–Vannucci model of rat neonatal HI at postnatal day 7 (PN7). Mitochondrial and lysosomal morphology and degree of co-localization were determined by immunofluorescence in the cerebral cortex. No difference in mitochondrial abundance was detected in the cortex after HI. However, net mitochondrial fission increased in both hemispheres of female brain, but was most extensive in the ipsilateral hemisphere of male brain following HI. Basal autophagy, assessed by immunoblot for the autophagosome marker LC3BI/II, was greater in males suggesting less intrinsic reserve capacity for autophagy following HI. Autophagosome formation, lysosome size, and TOM20/LAMP2 co-localization were increased in the contralateral hemisphere following HI in female, but not male brain. An accumulation of ubiquitinated mitochondrial protein was observed in male, but not female brain following HI. Moreover, neuronal cell death with NeuN/TUNEL co-staining occurred in both hemispheres of male brain, but only in the ipsilateral hemisphere of female brain after HI. In summary, mitophagy induction and neuronal cell death are sex dependent following HI. The deficit in elimination of damaged/dysfunctional mitochondria in the male brain following HI may contribute to male vulnerability to neuronal death and long-term neurobehavioral deficits following HIE. © 2016 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: mitophagy, sex-dependent, cell death, morphology, mitochondria, lysosome.

INTRODUCTION

Neonatal hypoxic-ischemic encephalopathy (HIE) is a sexually dimorphic brain disorder affecting approximately 1.5–2/1000 live births (Kurinczuk et al., 2010;Davidson et al., 2015). Males are particularly vulnerable to adverse long-term outcome compared to females affected by HIE (Hill and Fitch, 2012; Smith et al., 2014), but sex-dependent pathophysiological mechanisms are not well understood. No sex differences in lesion volume have been found following HI in rats (Smith et al., 2014), but there is one recent report of increased lesion volume in male compared to female mice following HI (Mirza et al., 2015). Cell death proclivity in several brain injury models is sexually dimorphic (Du et al., 2004; Li et al., 2005, 2009, 2011; Yuan et al., 2009; Siegel et al., 2011; Manwani and McCullough, 2011; Hill et al., 2011a; Hill and Fitch, 2012; Siegel and McCullough, 2013) and a sex difference in cell death was also recently reported in moderate, but not severe hypoxia–ischemia (HI) injury in postnatal day 7 (PN7) rats (Askalan et al., 2015). The large body of clinical evidence demonstrating adult females have a better outcome after stroke than similarly aged males is commonly attributed to the presence of estrogen (reviewed in Turtzo and McCullough, 2010). In contrast, the mechanisms underlying neonatal sex differences in neurobehavioral outcome, when brain hormone levels are equivalent (Konkle and McCarthy, 2011), are not well understood. While hormone concentrations are similar, there is evidence that sex differences in androgen modulation of GABAergic neurotransmission may influence the susceptibility to neonatal brain injury (Nunez and McCarthy, 2008). Despite this growing evidence, sex differences in clinical studies of perinatal brain injury remain largely underrepresented.

*Correspondence to: G. Fiskum, University of Maryland School of Medicine, 685 West Baltimore Street, Bldg. MSTF 5-34, Baltimore, MD 21201, USA. Fax: +1-410-706-2550.
E-mail address: gfiskum@anes.umm.edu (G. Fiskum).
Abbreviations: HI, hypoxia–ischemia; HIE, hypoxic-ischemic encephalopathy; LAMP2, lysosome-associated membrane protein 2; LC3BI/II, microtubule-associated proteins 1A/1B light chain 3B; MCAO, middle cerebral artery occlusion; MQC, mitochondrial quality control; PN, postnatal day; TOM20, translocase of outer membrane 20; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling.

It is well established that mitochondrial dysfunction and oxidative stress contribute to cell death following neonatal HI injury (Blomgren and Hagberg, 2006; Niatsetskaya et al., 2012; Ten and Starkov, 2012). We recently reported a male susceptibility to mitochondrial respiratory dysfunction and oxidative damage following HI (Demarest et al., 2016). These findings support the hypothesis that a mitochondrial mechanism may underlie the sex differences in long-term neurobehavioral outcome observed following HI. Mitochondrial fission (or fragmentation) (Pradeep et al., 2014; Zhang et al., 2015; Owens et al., 2015a) and autophagy (Weis et al., 2014; Li et al., 2015; Yu et al., 2015) are known to occur following cerebral ischemia–reperfusion injury. It is hypothesized that mitochondrial fragmentation segregates oxidatively modified mitochondrial proteins for elimination via mitochondrial-specific autophagy (mitophagy) (Twig et al., 2008; Soubannier et al., 2012; Norton et al., 2014). Increases in autophagy following ischemia–reperfusion injury are well recognized. However, there are conflicting reports that increased autophagy following cerebral ischemia–reperfusion injury can be detrimental (Baek et al., 2014; Yang et al., 2015; Au et al., 2015; He et al., 2016; Xie et al., 2016) or neuroprotective (Carloni et al., 2012; Qi et al., 2014; Su et al., 2014; Wang et al., 2014; Jiang et al., 2015). Sex differences in autophagy both *in vitro* (Du et al., 2009) and *in vivo* following HI (Weis et al., 2014) have been reported. However, the role of mitophagy following neonatal HI is unknown. Thus, in the current study, we tested the hypothesis that mitophagy is sexually dimorphic and neuroprotective 20–24 h following neonatal HI.

EXPERIMENTAL PROCEDURES

Animals

All animal procedures were approved by the University of Maryland Institutional Animal Care and Use Committee in accordance with the NIH Guide for the Care and Use of Laboratory Animals. $N = 146$ total postnatal day 8 (PN8) Sprague–Dawley rat pups from timed pregnant females were obtained from Charles River Laboratories (Wilmington, MA) for this study. Of these, $n = 48$ male, and $n = 51$ females were used for 6–11 individual brain mitochondria isolations per group as previously described (Demarest et al., 2016). The remaining pups were allocated to the following groups for biochemical measures and histology: Sham $n = 10$ male, $n = 11$ female; HI: $n = 14$ male, $n = 12$ female. Some tissues used in this study were derived from the same cohort of animals used in a previous study (Demarest et al., 2016).

Western blot

Total brain homogenate (25 μg protein per sample) was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) using the BioRad tetra cell system and AnyKd® gels (BioRad, Hercules, CA, USA). Gels were transferred to 0.2 μm pore size PVDF via the Transblot Turbo semidry transfer system (BioRad). Blots were blocked in 5% milk in Tris-buffered saline (TBS) plus 0.1% tween-20 and incubated overnight at 4 °C with outer mitochondrial membrane marker TOM20 (1:2000; Santa Cruz, Dallas, TX, USA), autophagosome marker LC3BI/II (1:1000; Cell Signaling, Danvers, MA, USA) or β-actin (1:2000; Sigma, St. Louis, MO, USA) and washed 3× 10 min in TBS plus 0.1% tween-20 (TBST) and incubated in goat anti-rabbit-HRP secondary (1:5000) in 5% milk in TBST. Blots were visualized using Amersham (GE healthcare, Little Chalfont, UK) chemiluminescent substrate and scanned for densitometry analysis on the Digit imaging system (LiCor, Lincoln, NE, USA). Densitometry was normalized to β-actin. $n = 4$–6/group.

Dot blot

Brain mitochondria were isolated from rat pups as previously described (Demarest et al., 2016). Isolated brain mitochondria (2.5 μg) were dotted onto nitrocellulose membrane using the Bio-Dot® SF Microfiltration Apparatus (BioRad) according to the manufacturer's instructions. Nitrocellulose membranes were blocked for 1 h in TBST + 5% milk, washed in TBST and incubated in TBST + 5% milk plus anti-ubiquitin antibody (1:1000; Cell Signaling) overnight at 4 °C, washed 6× 10 min in TBS plus 0.1% tween-20 (TBST) and incubated in goat anti-rabbit-HRP secondary (1:10,000) in 5% milk in TBST. Blots were visualized with SuperSignal™ West Femto (ThermoFisher, Waltham, MA, USA) on BioRad Chemi-doc™ MP imaging system. The chemiluminescent signal was calculated in relative light units (RLU) per μg of protein. $n = 6$–11/group.

Fluorescence immunohistochemistry

Forty-micron thick brain sections were cut and mounted as previously described (Demarest et al., 2016). Following a thirty-minute permeabilization in TBS + 0.03% Triton-X (TBSTx), sections were co-incubated in TOM20 (SantaCruz) and LAMP2 (SantaCruz) primary antibodies in IHC slide holders (Millipore, Billerica, MA, USA) for 72 h at 4 °C. Slides were washed in TBSTx 6 times for 10 min prior to a 72-h incubation with secondary antibodies; donkey anti-rabbit alexa 488 ReadyProbes® (Invitrogen, Carlsbad, CA, USA) and Donkey anti-Goat IgG (H + L), Alexa Fluor® 555 conjugate (ThermoFisher). Slides were removed from IHC slide Holders and washed in TBSTx 6 times for 10 min and cover-slipped using DAPI containing mounting media (DAPI Flouromount-G®; SouthernBiotech, Birmingham, AL, USA) prior to imaging. $n = 4$/group.

TOM20/LAMP2 image acquisition and analysis

Images were acquired at 63.3x oil magnification on a Zeiss Axioimager M2 microscope using the apotome. Images of three brain sections containing CA1 hippocampus were captured to ensure consistent cortical area between animals. Three images of cortical layers II/III per hemisphere, in each section, were imaged to ensure sufficient sampling of the cortex and eliminate any potential selection bias. A total of 9