Sexually Dimorphic Impairment of Brain Mitochondrial Respiration Following Neonatal Hypoxic-Ischemia

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Introduction

Neonatal brain injury including neonatal hypoxia-ischemia (HI) is a common cause of lifelong neurological deficits (1). Currently, hypoxia is the sole treatment available and is only partly neuroprotective. Traumatic brain injury (TBI) (2) and neonatal hypoxic bienergetics, which may contribute to cell death and neurologic injury. Acetyl-Carnitine neuroprotective in pediatric TBI models (6,7), possibly by protection against bioenergetic dysfunction. Anecdotal clinical reports and preclinical research on animal models of neonatal HI suggest males are more vulnerable to HI than females (3). Based on these observations, we tested the hypothesis that male rat pups exhibit greater brain mitochondrial respiratory impairment than females following the Rice-Vannucci model of neonatal HI.

Methods

Rice-Vannucci model of HI (Rice et al., 1981). Neonatal HI (PND 7) pups weighing between 12-15 g were exposed to 8% ambient CO2 for 75 min following ligation of the right carotid artery under sodium pentobarbital anesthesia. ALCAR treatments (20 mg/kg) were initiated subcutaneously immediately and 4 h following hypoxic-ischemia.

Mitochondrial Isolation: At 20 hr following HI, 3 bilateral and 3 contralateral cerebral hemispheres were respectively pooled and used for isolation of synaptic plus non-synaptic mitochondria using the protocol described in the ‘Rice-Vannucci method’ (5). The investigator was blinded to the identity of treatment vs. control rats (6-8/group).

Clark Electrode Respirometry: Brain mitochondrial respiration was measured using an O2 electrode chamber equipped with magnetic stirring and containing isometric medium maintained at 37°C containing 5 mM pyruvate plus 5 mM malate (Complex I or 5 mM Succinate (Complex II). In the presence of Complex II activity, attached increases in respiration were initiated by the addition of ADP (10-20 µM), rotenone (1 µM), and oligomycin, an inhibitor of the ATP synthase. Then, maximal uncoupled respiratory rates were measured by addition of protonophore FCCP.

Western Blot: 5 µg of freeze/thawed mitochondrial samples were used for western blots of representative proteins presented in oxidative phosphorylation complexes (Mitochondria 1 Subunit ND1/ND2; Mitochondria 2 Subunit ND4/ND4L; Mitochondria 3 Subunit Cytochrome, Complex 4 Subunit ATP synthase subunit alpha (SOD: CI subunit - 185D; CI subunit Core 2 - 170D; IV subunit - 1; 384D; ATP synthase subunit alpha - 165D; CI subunit - 165D; CI subunit Core 2 - 150D; CI subunit Core 2 - 143D; NADH oxidase - 163D; Complex 5 Subunit - 143D; Complex 6 Subunit - 131D; Complex 6 Subunit - 131D; Complex 6 Subunit - 123D; Complex 6 Subunit - 117D). Immobilon-P polyvinylidene difluoride (Millipore, Billerica, MA) was used as the PVDF membrane. Blots were blocked with 5% non-fat dry milk in Tris-buffered saline containing 0.1% Tween-20. The following antibodies were used: Complex II subunit-α (Abcam, Cambridge, MA; #ab35510; 1:500), complex III subunit-γ (Abcam, #ab4533; 1:500), complex IV subunit-α (Abcam, #ab308; 1:500), complex V subunit-α (Abcam, #ab9240; 1:500), and complex VI subunit-β (Abcam, #ab3787; 1:500). Blots were incubated with appropriate secondary antibodies and visualized with the Western Lightning Chemiluminescence kit (PerkinElmer Life Sciences, Waltham, MA). The results were evaluated via densitometry using ImageJ software (version 1.51a; National Institutes of Health, Bethesda, MD). 

Novel Object Recognition Behavior: On PND 31 and 32, rats were acclimated to the arena. The following day, rats were presented with 2 identical objects and allowed to explore for 5 min. One hour later, same object was swapped for a novel object. Discrimination ratio was then calculated as (Time spent with novel object - Time spent with familiar object) / Total time spent exploring objects. 

Data Analysis: 2-way ANOVA with Fisher’s post hoc test were performed for respiration data analysis and One-way ANOVA with Bonferroni post hoc test was performed for Western blot and behavioral analyses.

References


Conclusions

- State 3 and uncoupled respiration is inhibited at 20 h following HI using mitochondria from either male or female pups.
- Respiratory inhibition following HI is observed in the presence of either Complex I or II respiratory substrates.
- Males but not females exhibit mitochondrial respiratory impairment in the contralateral, hypoxia alone hemispheres.
- Hypoxia alone mitochondria from males treated with ALCAR do not exhibit respiratory impairment.
- No selective decline in oxidative phosphorylation protein subunits is observed at 20 h following HI.
- Only males exhibit impaired novel object recognition after HI, which is not observed following treatment with ALCAR.
- Studies are in progress to determine the mechanism of sexually dimorphic impairment of mitochondrial respiration that occurs after HI.

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