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Mitochondrial response in a toddler-aged swine model following diffuse non-impact traumatic brain injury

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ABSTRACT

Traumatic brain injury (TBI) is an important health problem, and a leading cause of death in children worldwide. Mitochondrial dysfunction is a critical component of the secondary TBI cascades. Mitochondrial response in the pediatric brain has limited investigation, despite evidence that the developing brain's response differs from that of the adult, especially in diffuse non-impact TBI. We performed a detailed evaluation of mitochondrial bioenergetics using high-resolution respirometry in a swine model of diffuse TBI (rapid non-impact rotational injury: RNR), and examined the cortex and hippocampus. A substrate-uncoupler-inhibitor-titration protocol examined the role of the individual complexes as well as the uncoupled maximal respiration. Respiration per mg of tissue was also related to citrate synthase activity (CS) as an attempt to control for variability in mitochondrial content following injury. Diffuse RNR stimulated increased complex II-driven respiration relative to mitochondrial content in the hippocampus compared to shams. LEAK (State 4_o) respiration increased in both regions, with decreased respiratory ratios of convergent oxidative phosphorylation through complex I and II, compared to sham animals, indicating uncoupling of oxidative phosphorylation at 24 h. The study suggests that proportionately, complex I contribution to convergent mitochondrial respiration was reduced in the hippocampus after RNR, with a simultaneous increase in complex-II driven respiration. Mitochondrial respiration 24 h after diffuse TBI varies by location within the brain. We concluded that significant uncoupling of oxidative phosphorylation and alterations in convergent respiration through complex I- and complex II-driven respiration reveals therapeutic opportunities for the injured at-risk pediatric brain.

1. Introduction

Traumatic brain injury (TBI) is an important health problem and is set to become the third leading cause of death and disability in the world by 2020 (Coronado et al., 2011; Gean and Fischbein, 2010).

Diffuse TBI triggers a heterogeneous insult to the brain induced by traumatic biomechanical shearing forces when the head is rapidly accelerated and/or decelerated, such as during player-to-player contacts in sports settings, impacts after falls, or whiplash injuries in car crashes. Axonal shear stretch leads to the opening of voltage-gated calcium channels that, ultimately, precipitates mitochondrial dysfunction, bioenergetic failure, and the release of secondary messengers that end in apoptosis and death (Balan et al., 2013; Glenn et al., 2003; Lifshitz et al., 2003; Marcoux et al., 2008; Ragan et al., 2013; Xu et al., 2010).Thus, mitochondria play a central role in cerebral metabolism and regulation of oxidative stress, excitotoxicity, and apoptosis in acute brain injury; however, the mechanistic response and time course following diffuse TBI, especially in the immature brain at differing developmental stages, has limit investigation (Balan et al., 2013; Gilmer et al., 2010; Lifshitz et al., 2004; Robertson et al., 2009). Furthermore, the challenge of extrapolating adult models of diffuse TBI to pediatric models includes developmental differences in biomechanical properties and biological responses that vary in the infant, toddler, adolescent, and adult (Grate et al., 2003; Ibrahim et al., 2010; S. Sullivan et al., 2015; Weeks et al., 2014). In addition, there are critical differences in mitochondrial characteristics in the developing brain as it matures, such as the number and density of complexes of the electron transfer chain,

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antioxidant enzyme activity and content, and lipid content (Bates et al., 1994; Del Maestro and McDonald, 1987). Taken together, the immature brain's response to TBI changes during development from infancy through adolescence and differs with injury mechanism (Armstead, 2005; Duhaime, 2006; Duhaime et al., 2000; Durham and Duhaime, 2007). These unique features of the developing brain underscore the importance of characterizing the bioenergetic failure and cell death cascades following TBI in the immature brain in order to develop age-specific mitochondrial-directed neuroprotective approaches.

Previously we reported differences in the regional mitochondrial responses in neonatal piglets, age 3–5 days, following diffuse white matter injury using our large animal model (Kilbaugh et al., 2011). In our current investigation, we have expanded our investigation to the 4-week old animals with comparable neurodevelopment to a human toddler. Furthermore, we expanded our previous techniques to investigate functional mitochondrial respiration, within integrated mitochondrial networks of fresh brain tissue, to focus on pathologic metabolic pathways following TBI.

2. Materials and methods

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, and was approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania (Number: 803401). Four-week old (8–10 kg) piglets, with comparable neurodevelopment to a human toddler, were studied (Armstead, 2005; Duhaime, 2006). In an effort to limit heterogeneity, only females were used based on our prior work (Missios et al., 2009). Twenty-eight piglets were randomly assigned to sham or injury cohorts, consisting of a single rapid non-impact rotational injury in the sagittal plane ($n = 18$ injured-RNR, $n = 10$ naïve sham-RNR). Animals were sacrificed 24 h after TBI.

2.1. Animal preparation

Anesthetic regimen included: 1) premedication with an intramuscular injection of ketamine (20 mg/kg) and xylazine (2 mg/kg), 2) induction: 4% inhaled isoflurane in 1.0 fraction of inspired oxygen via snout mask, until abolishment of response to a reflexive pinch stimulus, 3) maintenance: 1% inhaled isoflurane via endotracheal tube with fraction of inspired oxygen to 0.21. Buprenorphine (0.02 mg/kg) was also delivered intramuscularly for analgesia prior to injury. A circulating water blanket kept core body temperature constant between 36 and 38 °C (monitored via a rectal probe), and non-invasive blood pressure, oxygen saturation, heart rate, respiratory rate, and end-tidal CO_2 were continuously monitored throughout the experiment (Surgivet Advisor V9204; Smith Medical, Waukesha, WI).

2.2. Rapid non-impact rotational (RNR) injury

Diffuse closed head TBI was induced using an established rapid head rotation technique described previously (Eucker et al., 2011; Ibrahim et al., 2010; Raghupathi et al., 2004). While maintained on isoflurane, the head of the piglet was secured to a bite plate by a snout strap. Isoflurane was withdrawn immediately prior to injury, and the head was rotated rapidly (10–15 ms) ventral-to-dorsal in the sagittal plane with the center of rotation at the cervical spine. The peak angular velocity was nearly constant across the injured group, averaging 126 ± 0.72 rad/s.

Immediately after the RNR, the animal was removed from the injury device. At this angular velocity, rotation direction, and age animals experienced a brief period of hypoactivity, irritability and gait instability. Animals did not experience apnea or hemodynamic instability following TBI.

Following RNR, animals had significant neurologic deficits, including lethargy and longer periods of recumbancy, and unsteady gait compared to shams. However, animals eventually were able to vocalize, ambulate, maintain body temperature and exhibit proper feeding and drinking behaviors. These injuries are best described as mild-to-moderate in severity, based on parallels with human clinical severity classifications (Adelson et al., 2012; Miller et al., 2012) and neuropathology findings 6 days after RNR (Weeks et al., 2014).

2.3. Preparation of tissue homogenates

At 24 h post-RNR, a craniotomy was performed and a 2 cm^2 region of left frontal cortex was resected and both hippocampal regions extracted rapidly (less than 10 s) and combined. As a starting point for our initial large animal studies we chose 24 h as our terminal time-point for two critical reasons: 1) we have documented significant injury with neuropathology, imaging and behavior at this time point in our model of diffuse TBI (Jaber et al., 2015; Kilbaugh et al., 2011, 2015; Weeks et al., 2014), and 2) other investigators have documented bioenergetic and mitochondrial alterations in rodents following diffuse TBI at this particular time point. The hippocampus and cortex were studied as the areas of interest for two critical factors: 1) previous studies from our laboratory have documented significant neuropathology and mitochondrial dysfunction within these regions following RNR (Coats and Margulies, 2006; Eucker et al., 2011; Kilbaugh et al., 2011; Weeks et al., 2014), and 2) if our hypothesis was consistent with previous findings in adults and infants, and these areas of interest do exhibit alterations in mitochondrial functional pathways, then these findings would be instrumental in the evaluation of neurobehavioral outcomes in future studies, linking mitochondrial bioenergetics and long-term outcomes (S. Sullivan et al., 2013). Following extraction, tissue was placed immediately in ice-cold isolation buffer (320 mM sucrose, 10 mM Trizma base, and 2 mM EGTA). Blood and vasculature was dissected and 1 mg of wet weight tissue was gently homogenized on ice in MiR05 buffer (110 mM sucrose, 0.5 mM EGTA, 3.0 mM $MgCl_2$, 60 mM K-lactobionate, 10 mM KH_2PO_4, 20 mM taurine, 20 mM HEPES and 1.0 g/l fatty acid-free BSA) using a 5 ml Potter–Elvehjem teflon-glass homogenizer to a concentration of 1 mg wet weight tissue/10 μl MiR05 buffer.

2.4. Mitochondrial high-resolution respirometry (HRR)

High-resolution Oxygraph-2 k (Oroboros Instruments, Innsbruck, Austria) was used to measure mitochondrial respiration. The instrument was calibrated daily, as previously described, and respiration measurements were obtained at a constant 37 °C with the addition of tissue homogenates to a final concentration of 1 mg per ml of Mir05 buffer (Kilbaugh et al., 2015). Oxygen consumption and oxygen flux were recorded using DatLab software (5.1, Oroboros Instruments, Innsbruck, Austria).

A substrate, uncoupler, inhibitor titration (SUIT) protocol previously used was specifically designed for porcine brain tissue (Kilbaugh et al., 2015). Complex specific substrates and inhibitors allowed for the assessment of respiratory capacities of the integrated electron transport system (ETS) (Fig. 1). Complex I (CI), and complex II (CII) respiratory capacities in brain tissue were evaluated separately; as well as with pathways of convergent electron input through the Q-junction (CI + II) using succinate and nicotinamide adenine dinucleotide (NADH)-linked substrates (Gnaiger, 2009). An optimized dose of digitonin, 1 μl (50 mg/ml), necessary to achieve accurate and consistent oxidative phosphorylation capacity within synaptasomes using water-soluble substrates, and achieve similar results in brain tissue homogenates and isolated brain mitochondria (Kilbaugh et al., 2015, 2011). Exogenously administered cytochrome c did not induce a significant effect on mitochondrial respiration at the optimal digitonin dose, indicating an intact outer mitochondrial membrane (data not shown) (Brustovetsky et al., 2002; Sims and Blass, 1986). Sequential additions