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Sex dependent mitochondrial respiratory impairment and oxidative stress in a rat model of neonatal hypoxic-ischemic encephalopathy

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Abstract

Increased male susceptibility to long-term cognitive deficits is well described in clinical and experimental studies of neonatal hypoxic-ischemic encephalopathy. While cell death signaling pathways are known to be sexually dimorphic, a sex-dependent pathophysiological mechanism preceding the majority of secondary cell death has yet to be described. Mitochondrial dysfunction contributes to cell death following cerebral hypoxic-ischemia (HI). Several lines of evidence suggest there are sex differences in the mitochondrial metabolism of adult mammals. Therefore, this study tested the hypothesis that brain mitochondrial respiratory impairment and associated oxidative stress is more severe in males than females following HI. Maximal brain mitochondrial respiration during oxidative phosphorylation was two-fold more impaired in males following HI. The endogenous antioxidant glutathione was 30% higher in the brain of sham females compared to males. Females also exhibited increased glutathione peroxidase (GPx) activity following HI injury. Conversely, males displayed a reduction in mitochondrial GPx4 protein levels and mitochondrial GPx activity. Moreover, a 3 to 4-fold increase in oxidative protein carbonylation was observed in the cortex, perirhinal cortex, and hippocampus of injured males, but not females. These data provide the first evidence for sex dependent mitochondrial respiratory dysfunction and oxidative

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Author Contributions

All authors were directly involved in the design or conduct of the study. TGD was responsible for data collection, analysis, interpretation, and manuscript/figure preparation. RAS assisted in data collection and manuscript preparation. JW performed all surgeries and drug treatments. GF and MCM were responsible for experimental design and interpretation of results. All authors contributed to editing the manuscript.

Disclosure/conflict of interest

The authors declare no conflict of interest

damage which may contribute to the relative male susceptibility to adverse long-term outcomes following HI.

Keywords

Glutathione; Glutathione peroxidase; protein carbonyl; oxidative phosphorylation

Introduction

Neonatal hypoxic-ischemic encephalopathy (HIE) is a major cause of lifelong motor and cognitive impairment, affecting approximately 1.5-2/1000 live births (Kurinczuk *et al.* 2010; Davidson *et al.* 2015). Meta-analysis of clinical data indicates human male infants suffer greater long term IQ impairment than similarly injured females following HIE (Smith *et al.* 2014). Preclinical research utilizing the Rice-Vannucci rodent model of neonatal hypoxic-ischemia (HI) also reveals a male susceptibility to behavioral deficits in cognitive tasks compared to similarly injured females (Smith *et al.* 2014, Hill and Fitch 2012). Notably, cell death signaling cascades are sexually dimorphic following neonatal HI, with caspase-dependent and caspase-independent cell death proclivity in female and male rat pups, respectively (Hill and Fitch 2012; McCullough *et al.* 2005). Despite these advances in knowledge, sex differences in pathophysiological processes that precede the majority of secondary cell death have yet to be identified.

It is well established that mitochondrial dysfunction and oxidative stress occur following ischemia/reperfusion injury (Blomgren and Hagberg 2006) and that these coincide with the majority of cell death, during secondary injury, over days following injury (Ferriero 2001). Moreover, adult animal and *in vitro* studies suggest sex differences in mitochondrial mechanisms known to be involved in brain injury including reactive oxygen species (ROS) generation and antioxidant detoxification capacity (reviewed in Demarest and McCarthy 2015). The immature brain is known to be particularly susceptible to oxidative stress-mediated ischemia/reperfusion injury compared to the mature brain. This age dependent difference is due, in part, to a reduced antioxidant capacity in the developing brain (Blomgren and Hagberg 2006; Hagberg *et al.* 2009). These data point to the involvement of a mitochondrial mechanism in the pathophysiology of HI as mitochondria are the major generators of ROS and the gatekeepers of cell death initiation.

Mitochondrial respiratory impairment is implicated in the pathophysiology of nearly all acute central nervous system (CNS) injuries including traumatic brain injury (Robertson *et al.* 2009), spinal cord injury (Patel *et al.* 2012), adult cerebral ischemia (Fiskum 2000; Blomgren *et al.* 2003) and neonatal HI (Niatetskaya *et al.* 2012; Puka-Sundvall *et al.* 2000). Moreover, mitochondria have recently been recognized as a pivotal hub of injury response in the developing brain (Hagberg *et al.* 2014) and are increasingly targeted for neuroprotective drug development. While mitochondrial respiratory dysfunction and oxidative stress are known to occur following HI, experiments reporting these findings were performed solely in male animals or animals of unidentified sex (Niatetskaya *et al.* 2012; Ten *et al.* 2010). Thus,

it is still unknown if mitochondrial respiratory impairment and generation or detoxification of ROS occurs in a sex dependent manner.

Defending against increasing ROS during times of injury is particularly important to preserve neuronal survival and mitigate adverse long term outcomes (Starkov *et al.* 2004). Endogenous antioxidant defenses systems play a critical role in the maintenance of redox homeostasis (Rodriguez-Rodriguez *et al.* 2014). Among these, the glutathione dependent antioxidant system is arguably one of the most important cellular defense mechanisms. Reduced glutathione (GSH) is a low molecular weight thiol essential for maintaining redox state by providing reducing equivalents to oxidized molecules via spontaneous reduction reactions and as a substrate for antioxidant enzymes like glutathione peroxidases. Glutathione peroxidase (GPx) enzymes are present in many subcellular compartments, including the mitochondria (Ursini *et al.* 1985; Ursini *et al.* 1986; Mbemba *et al.* 1985). GPx antioxidant capacity is known to be affected by HI injury. For example, hypoxic preconditioning up-regulates GPx activity and overexpression of human GPx1 reduces injury following HI (Sheldon *et al.* 2007). A recent study of pediatric traumatic brain injury determined that GSH is higher in mitochondria from uninjured females than males (Robertson and Saraswati 2015). Taken together, these observations suggest mitochondrial dysfunction and ROS detoxification/generation may be sex dependent following pediatric brain injury.

Acetyl-L-carnitine (ALCAR) is a naturally occurring endogenous compound with demonstrated antioxidant and neuroprotective properties in the immature and mature brain, following either ischemic or traumatic brain injury (Liu *et al.* 1993; Dell'Anna *et al.* 1997; Zanelli *et al.* 2005; Scafidi *et al.* 2010b; Goo *et al.* 2012; Rosenthal *et al.* 1992; Xu *et al.* 2015). Although the exact mechanism of action is incompletely understood, ALCAR may afford neuroprotection by acting as an antioxidant or as an alternative biofuel via donation of acetyl groups for the synthesis of acetyl-coA and/or by increasing L-carnitine mediated shuttling of fatty acids into the mitochondria for beta oxidation (Jones *et al.* 2010; Scafidi *et al.* 2010a).

Given the evidence that males are particularly susceptible to neonatal HI, we tested the hypothesis that mitochondrial respiratory impairment and oxidative stress mediated damage contributes to this sex biased vulnerability. We further assessed whether ALCAR reduces mitochondrial dysfunction and oxidative stress following neonatal HI.

Materials and Methods

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=198 total rat pups were used in this study; 174 pups were used for collection of brain homogenates and isolation of brain mitochondria. Brains from three pups were pooled for mitochondria isolation, resulting in 58 separate mitochondria isolations (sham: n=9 male, n=9 female; HI: n=9 male, n=8 female, HI + ALCAR: n=11 male, n=12 female). The other 24 pups were perfusion fixed for histology.

Neonatal Hypoxic-Ischemia

Litters were culled to nine pups on the day of birth; postnatal day (PN) 0. HI was performed using a modification of the Rice-Vannucci model (Xu *et al.* 2015; Rice, III *et al.* 1981). HI results in an ipsilateral hypoxic-ischemic hemisphere and a contralateral “hypoxia-only” hemisphere. Briefly, male and female postnatal day 7 (PN7) Sprague-Dawley rat pups weighing between 12.5–15.5 g were randomly assigned to sham, HI, or HI+ALCAR groups. Anesthesia was induced with 3% isoflurane for one to two minutes and maintained under 1.5% isoflurane on a heating pad (37°C) during the surgical procedure. The right carotid artery was surgically exposed and severed midway between two sterile ligatures. The skin incision was sutured and the pups allowed to recover consciousness during 25 min in a container immersed in a 37°C water bath and then returned to their dam for 60 min. Pups were then placed in warmed, humidified jars, and exposed to 8% O₂ for 75 min. Pups recovered in jars in the warm water bath for another 2 hr before returning to the home cage. ALCAR (100 mg/kg, subcutaneously, in 8% NaHCO₃ in phosphate buffered saline) or equal volume phosphate buffered saline treatments were administered immediately after, and 4 hr after hypoxia. Sham animals received the same duration of anesthesia and the neck incision but no carotid artery ligation or hypoxia.

Brain mitochondria isolation

Mitochondrial isolations were performed 20 hours following HI. For each mitochondrial preparation, three male or female 8 day old Sprague-Dawley rat pups were euthanized by decapitation, cerebellum and olfactory bulbs removed and the left and right hemispheres rapidly removed for separate isolation of synaptic plus non-synaptic mitochondria by a modification of the previously described “digitonin” method (Scafidi *et al.* 2010b). Following euthanasia, cortical hemispheres were rapidly removed and minced in 12 ml ice-cold mannitol-sucrose (MS) buffer pH 7.4 (225 mM mannitol, 75 mM sucrose, 5 mM Hepes, 1 mg/ml fatty-acid-free bovine serum albumin (BSA), 1 mM EGTA). This was rapidly repeated for each of the 3 animals, separately pooling 3 ipsilateral and contralateral hemispheres, respectively, per preparation. n=4–6 independent preparations per treatment group. The brain tissue was homogenized manually using 10 strokes of a Potter-Elvehjem teflon tissue grinder pestle (Wheaton Science Products, Millville, NJ) in a 15 ml Dounce Homogenizer (Bellco glass, Vineland, NJ). The homogenate was centrifuged at 1300 × g for 3 min at 4°C. The supernatant was removed and kept on ice and the pellet was resuspended in 3 ml MS and centrifuged at 1300 × g. Resulting supernatants were pooled and centrifuged at 22,000 × g for 8 min. The pellet, containing primarily free (non-synaptic) mitochondria plus synaptosomes, was then resuspended in 10 ml of MS buffer, using gentle trituration with a disposable Pasteur pipette. Twenty µl of 10% (wt/vol) digitonin (Calbiochem) in dimethylsulfoxide (DMSO) was added and gently mixed on ice for 3 min. This suspension material was spun at 22,000 × g for 8 min. The appearance of the pellet after this centrifugation following the addition of digitonin is typically more homogeneous and dense than the appearance of the pellet prior to this step. This change is the result of digitonin disrupting the synaptosomal plasma membranes and releasing synaptic mitochondria, resulting in a more pure mitochondrial pellet. Nevertheless, a small beige “fluffy layer” of material is present above a dark brown pellet. The supernatant and fluffy layer were carefully removed by aspiration with a glass Pasteur pipette attached to a vacuum line. The

mitochondrial pellet was resuspended to a final volume of 1.5 ml of MS buffer and centrifuged at $22,000 \times g$ for 10 min at 4°C in a microfuge. The mitochondrial pellet was resuspended in MS without EGTA. Protein concentrations were determined by standard Bradford method employing a BSA standard curve.

Mitochondrial oxygen consumption

Equal amounts (0.5 mg protein/ml) of isolated mitochondria were added to a thermostatically-controlled O₂ electrode chamber (Hansatech Instruments, Norfolk, England) equipped with magnetic stirring and containing 0.5 ml of respiration buffer consisting of 70 mM sucrose, 220 mM mannitol, 5 mM KH₂PO₄, 5 mM MgCl₂, 1 mM EGTA, 0.2% fatty acid-free BSA, and 2 mM HEPES, pH 7.4, 37°C. Malate and pyruvate were used as substrates to determine respiration mediated by electron transport chain complexes I through IV and succinate plus rotenone, a complex I inhibitor, were used to assess respiration mediated by complexes II through IV. State 3 respiration was initiated by the addition of 1.0 mM ADP. Approximately 2 min following ADP addition, state 3 respiration was terminated and state 4_o respiration (resting) was initiated with addition of 1.25 µg/ml oligomycin, an inhibitor of mitochondrial ATP synthase. The ensuing rate of respiration, designated as state 4_o, is a reflection of the proton permeability of the inner membrane, without interference from ATP turnover due to the presence of ATP hydrolases. The maximal rate of uncoupled respiration was subsequently measured by addition of 60 nM carbonyl cyanide p-(trifluoromethoxy)phenylhydrazone (FCCP); a protonophore uncoupling molecule. An n=4–6 independent experiments were performed with separate preparations of isolated brain mitochondria.

Glutathione quantification

Reduced glutathione (GSH) was measured as previously described (Robertson and Saraswati 2015; Bayir *et al.* 2009) with the modification that sulfhydryl selective fluorophore ThioGloIV (Millipore, Billerica, MA) was used instead of ThioGlo1 (discontinued). Briefly, total brain homogenate was incubated with ThioGloIV (excitation 400nm) and emission at 465nm quantified in a 96-well fluorescence plate reader (FLUOstar OPTIMA, BMG Labtech). Following this initial reading, samples were incubated with glutathione peroxidase and cumene hydroperoxide to deplete all reduced glutathione and ThioGloIV emission at 465nm measured again. Subtracting the final reading from the initial reading yields total reduced glutathione (GSH). All samples were run in duplicate and quantified by comparison to a standard curve of pure reduced glutathione (Sigma, St. Louis, MO). n=6/group.

Glutathione peroxidase activity

Glutathione peroxidase activity was determined in total brain homogenate and isolated mitochondrial fractions using the glutathione peroxidase assay kit according to the manufacturer's instructions (Cayman chemical, Ann Arbor, MI). Each well of a 96 well plate contained sample in 50 mM Tris-HCl, pH 7.6, 5 mM EDTA, 1 mg/ml BSA, and a co-substrate mixture containing NAD(P)H, GSH and glutathione reductase. The enzyme reaction was initiated by the addition of cumene hydroperoxide and the rate of decreasing absorbance at 340 nm was recorded for 5 minutes. Activity is represented as nmol NAD(P)H consumed per minute per mg protein. n=6 /group.

Western blot

Twenty μ g of freeze/thawed isolated brain mitochondrial samples were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) using the BioRad tetra cell system and AnyKd® gels (BioRad, Hercules, CA). Gels were transferred to 0.2 μ m pore size PVDF via the Transblot Turbo semidry transfer system (BioRad). Blots were blocked for 2 hours in 5% milk in Tris-buffered saline (TBS) plus 0.1% tween-20 and incubated overnight at 4°C with glutathione peroxidase 4 primary antibody (Cayman Chemical; 1:1000 dilution), washed 3 times for 10 minutes 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 chemiluminescent substrate (GE healthcare) and scanned for densitometry analysis on the Digit imaging system (LiCor). Densitometry was normalized to a representative total protein (visualized by ponceau S staining) band of 62kD for each blot (Olesen and Auger 2005) and calculated as a percentage of sham animals to control for interblot variation. n=4/group.

Protein Carbonyl Immunohistochemistry

Brains were cut in a 1–12 series of 40 μ m sections on a cryostat (Leica). Each series contained approximately 6 sections (480 μ m apart) representing the entire brain. These were mounted to superfrost slides (Fisher Scientific). Protein carbonylation was then detected (Zheng and Bizzozero 2010; Lazarus *et al.* 2015). Briefly, following a thirty minute permeabilization in tris buffered saline (19.98 mM Tris, 136 mM NaCl, pH 7.4) containing 0.03% triton-x, DNPH derivitization was conducted (1mg/mL DNPH in 2N HCl) for 30 minutes in the dark, washed for one hour and incubated overnight at 4°C with anti-DNP antibody (Sigma). The following day, sections were washed and incubated with secondary antibody (Donkey anti-rabbit alexa 488 ReadyProbes® Invitrogen, Carlsbad, CA) for three hours in the dark. Following washing, brain sections were cover-slipped using DAPI containing mounting media (DAPI Fluoromount-G®, SouthernBiotech, Birmingham, AL). Images of contralateral and ipsilateral cortices (external granular and pyramidal layers II/III), perirhinal cortices, and CA1 hippocampi were captured in the 3 sections containing CA1 hippocampus from each brain under identical fluorescent parameters (100 ms exposure, 0 gain, 1.00 gamma) at 20x magnification on a Zeiss Axioimager M2 microscope. Specificity was verified using a naïve 400g adult male Sprague Dawley rat transcardially perfused with oxygenated artificial cerebrospinal fluid (140 mM NaCl, 3 mM KCl, 1.85 mM CaCl₂, 1.7 mM MgCl₂, 1.5 mM Na₂HPO₄, 0.14 mM NaH₂PO₄, pH 7.4) containing 50 μ M nitric oxide (NO) donor diethylenetriamine NO for 5 minutes prior to 4% paraformaldehyde perfusion as a positive control for oxidative stress. Integrated intensity measurements were calculated for each image in Image J software using the following thresholds (color: 0–120; saturation: 0–255, brightness: 15–255). Integrated intensities from each brain section were calculated and averaged per animal (n=4 animals/group). The integrated intensity in the positive control was greater than any in the HI groups (data not shown).

Data analysis

All results are represented as mean $\pm$ SEM. Parametric statistical analysis was performed using SigmaPlot version 12.0. Rates of mitochondrial respiration and glutathione

concentration were compared by two-way ANOVA with Sex and Group as factors. 'Group' was defined by surgical condition (Sham/HI), post-HI treatment (Saline/ALCAR) and Hemisphere (Contra/Ipsi) with Fisher's post hoc test to determine intergroup differences. Western blots were analyzed by one-way ANOVA for male and female samples, separately. Carbonyl immunohistochemistry was analyzed by 2-way ANOVA with Bonferroni correction. P-values < 0.05 were considered significant. Graphs were generated in GraphPad Prism 5.0 and Excel 2010.

Results

Sex dependent mitochondrial respiratory impairment

To test the functional impairment of brain mitochondria, oxygen consumption measurements were performed with isolated brain mitochondria from male and female littermates 20 hours following sham surgery or HI injury +/- administration of ALCAR. Mitochondrial complex I dependent respiration was measured using malate and pyruvate as electron donors (representative traces Fig. 1a, b). There was no difference between mitochondrial respiratory rates for either sex or hemisphere in sham animals. Mitochondrial complex I dependent state 3 (ADP stimulated) respiration was significantly more impaired in both hemispheres of male brain compared to female brain following HI. State 3 respiratory rates for mitochondria isolated from the contralateral hemisphere of males were 30% lower compared to sham animals while female brain mitochondria exhibited no respiratory impairment in this hemisphere. In the ipsilateral hemisphere, mitochondrial respiration from male brain was twice as impaired as respiration from the ipsilateral hemisphere from female brain. ALCAR treatment prevented the impairment of respiration by mitochondria from the contralateral hemisphere of males. Moreover, treatment of rats with ALCAR eliminated sex differences in respiratory inhibition. Mitochondrial state 3 respiration from the ipsilateral hemisphere was lower than mitochondrial respiration from the contralateral hemisphere in HI and HI +ALCAR groups of both sexes. There was no difference in mitochondrial state 4 respiration (ATP synthase-independent proton leak) from the contralateral hemisphere in either sex compared with mitochondria from contralateral hemispheres of shams. However, mitochondria from the contralateral hemisphere exhibited significantly higher state 4 rates than mitochondria from the ipsilateral hemisphere. In addition, females had a greater state 4 rate in mitochondria from the contralateral hemisphere in comparison to males following HI. ALCAR treatment reduced complex I dependent state 4 respiration in mitochondria from the ipsilateral hemisphere of female brain (Fig. 1 e, f). FCCP uncoupling of mitochondrial respiration closely resembled rates observed in state 3 respiration, with respiratory impairment of the ipsilateral hemisphere observed in both sexes (Fig 1. g, h). In HI males treated with ALCAR state 4 respiration was comparable to shams. Figure 1, i-j depicts the respiratory control ratio (RCR; state 3/state 4), an indicator of overall mitochondrial functional integrity. Mitochondria from sham animals had RCR values between 8 and 10; indicating intact and high functioning mitochondria. A significant decrease in the RCR of mitochondria isolated from both ipsilateral and contralateral hemispheres was observed solely in males following HI (Fig. 1i). To investigate if sex differences in mitochondrial respiratory impairment were specific to complex I, we also tested complex II dependent mitochondrial respiration.

Mitochondrial complex II dependent respiration was measured using succinate as the electron donor to succinate dehydrogenase (complex II) in the presence of complex I inhibitor rotenone, thus eliminating any flow of electrons through complex I of the electron transport chain. Complex II dependent state 3 respiration was impaired in mitochondria from both hemispheres of male and female brain following HI. As seen in complex I respiration, male brain mitochondrial respiratory inhibition in the contralateral hemisphere was not reduced following ALCAR treatment (Fig. 2a). whereas treatment with ALCAR after HI had no effect on state 3 respiration in females (Fig. 2b). Similarly, state 4 respiration was impaired in mitochondria from both hemispheres of male and females following HI. Complex II dependent state 4 respiration was significantly more diminished in ipsilateral brain mitochondria from males compared to ipsilateral female brain mitochondria. Treatment with ALCAR did not lead to any improvement of state 4 respiration in mitochondria from the ipsilateral side of either male or female brain. ALCAR treatment prevented the impairment of state 4 respiration, only in mitochondria from the contralateral hemisphere of males (Fig. 2 c, d). As seen in complex I dependent respiration, complex II dependent FCCP uncoupled respiration closely mirrored state 3 respiration. FCCP uncoupled respiration was inhibited in brain mitochondria from both hemispheres of males and females after HI. ALCAR prevented the impairment of contralateral FCCP uncoupled respiration in brain mitochondria from males and partially restored contralateral FCCP uncoupled respiration in brain mitochondria from females (Fig. 2 e, f). There were no differences in complex II dependent RCR following injury (Fig. 2 g, h). Since there were greater sex differences in complex I dependent respiration and complex I is known to produce ROS following HI in males (Niatetskaya *et al.* 2012), we hypothesized that the impairment in mitochondrial respiration observed may indicate sex differences in the production or detoxification of ROS following HI.

Sex dependent differences in antioxidant capacity

To determine putative sex differences in detoxification capacity of ROS in brain following HI, we assayed components of the well characterized glutathione antioxidant defense system. Quantification of GSH revealed that brain homogenates from female uninjured sham controls had ~30% more GSH compared to sham males (Fig. 3a, b). Following HI, a significant decrease in GSH was observed in both hemispheres of the female brain. In males, there was an unanticipated increase in GSH in the contralateral hemisphere in both HI and HI+ALCAR treated rats. However, there was no change in GSH levels in the ipsilateral hemisphere compared to brain from male shams. We hypothesized that because glutathione peroxidase (GPx) is a major antioxidant enzyme utilizing GSH as its substrate, a sex difference in the impairment of GPx function may explain the observed changes in GSH following HI injury. GPx activity in brain homogenate was assayed to determine if the sex differences following injury were due to differences in GPx activity. GPx activity was significantly increased in the ipsilateral hemisphere from female brains following HI and in female HI pups treated with ALCAR (Fig. 3f). There was no difference in overall GPx activity in the brain homogenate from male pups in either hemisphere or any treatment condition (Fig. 3c,e). However, since we observed a significant increase in GSH following injury in the contralateral hemisphere, we hypothesized that the increase in GSH in the

contralateral hemisphere of males after HI could be due to an impairment of a mitochondrial GPx.

Eight mammalian isozymes of glutathione peroxidase (GPx1–8) have been described (Ursini *et al.* 1985; Ursini *et al.* 1986). GPx1 and GPx4 are the best characterized and both are present within the mitochondrial and cytosolic subcellular compartments. GPx4 is the only GPx isozyme with an ability to detoxify lipid hydroperoxides (Ursini *et al.* 1985; Ursini *et al.* 1986). Western blot analysis revealed no change in GPx4 levels in brain mitochondria isolated from female sham, HI and HI+ALCAR rat pups. In contrast, there was a significant decrease in GPx4 immunoreactivity in mitochondria from the contralateral hemisphere of male pups after HI. GPx4 immunoreactivity was increased in mitochondria from the ipsilateral hemisphere of male rats treated with ALCAR after HI (Fig. 4a).

To assess whether the HI-dependent reduction in GPx4 immunoreactivity resulted in a decreased functional ability to detoxify oxidative damage, GPx enzyme activity was determined in isolated mitochondria. Mitochondrial GPx activity was significantly reduced in mitochondria from the contralateral hemisphere of male rats following HI (Fig. 4b). Treatment with ALCAR after HI prevented this impairment. There was no reduction in the brain mitochondrial GPx activity of females in any treatment group (Fig. 4b).

Sex dependent oxidative protein carbonylation in cortex and hippocampus

To determine if there is excessive production of ROS after HI, direct measures of oxidative damage were performed. A 3 to 4 fold increase in perinuclear accumulation of protein carbonyls was observed in males following HI in both hemispheres of the cortex (Fig. 5), perirhinal cortex (Fig. 6) and CA1 region of the hippocampus (Fig. 7). ALCAR treatment significantly reduced protein carbonyl formation in males following HI in both hemispheres of the cortex, perirhinal cortex, and hippocampus (Figs. 5–7). No significant differences in protein carbonyl levels were observed in female brains in any treatment group (Figs. 5–7).

Discussion

Collectively, the most important findings of the current study are the identification of sex differences in mitochondrial respiratory impairment (Fig. 1–2) and oxidative stress (Fig. 5–7) following HI. Mitochondrial impairment in males is paralleled by deficits in GPx antioxidant capacity (Fig. 3) and resultant increases in protein oxidation at 20–24 hours (Fig. 5–7); at the beginning of secondary HI injury. Secondary injury is characterized by increases in oxidative stress that lead to massive tissue loss and cell death from 24 hours to weeks following HI (Ferriero 2001; Thornton *et al.* 2012). The present study found a bilateral susceptibility to NADH dehydrogenase (complex I) dependent state 3 brain mitochondrial respiratory impairment and a significant decrease in the respiratory control ratio after HI in males. Our findings are consistent with recent studies reporting sex differences in mitochondrial metabolism following HI (Morken *et al.* 2014a; Weis *et al.* 2012). Morken *et al.* (2014) found differential alterations in oxidative metabolism in male and female brain at multiple time points with magnetic resonance spectroscopy after HI at PND 7. They reported a more striking decrease early after HI in males, but more prolonged differences in metabolism in the cortex of females (Morken *et al.* 2014b). Weis *et al.* (2012) report that

enzyme activity of electron transport chain (ETC) complexes I-III of postnatal day 8 male Wistar rats is significantly lower than females 18 hours following HI, with decreases in ETC activity in the ipsilateral cortex and hippocampus of both sexes (Weis *et al.* 2012). Collectively, these data suggest brain mitochondrial ETC impairment is greater in males than in females after HI. Complex I is a major site of ROS production and known contributor to ROS generation following HI (Niatetskaya *et al.* 2012; Ten and Starkov 2012).

In the current study, we found that brain mitochondria from female animals increase complex I dependent proton leak (state 4 respiration) across the inner mitochondrial membrane significantly more than brain mitochondria from males following HI (Fig. 1 e, f). One possible explanation for the increases in proton leak is an increase in mitochondrial uncoupling. Mitochondrial uncoupling can be mediated by a family of uncoupling proteins (UCPs) that are activated by free fatty acids (FFA) (Korshunov *et al.* 1998) and ROS (Echtay *et al.* 2002). The increase in uncoupling of mitochondria from female brain may be explained by a greater activation of UCPs following HI. It should be noted that our respiration buffer contains BSA (1mg/mL), which can inhibit FFA-induced uncoupling via UCPs (Sullivan *et al.* 2004). Therefore, it is possible that the changes in state 4 respiration we observe (Fig 1, 2) would be more profound in a respiration medium absent of BSA. Activation of UCPs has a demonstrated neuroprotective effect in ischemic injury such that overexpression of UCP2 protects thalamic neurons following global ischemia (Deierborg *et al.* 2008) and treatment with mitochondrial uncoupler 2,4-dinitrophenol reduces lesion volume and improves mitochondrial function by reducing oxidative stress (Korde *et al.* 2005). The increase in state 4 respiration in the female brain may be a compensatory response to reduce mitochondrial free radical generation following HI. It is also possible that increased leakage of electrons from redox sites in the electron transport chain to oxygen, forming superoxide, could contribute to the increases in state 4 respiration.

Mitochondria are main sites of ROS generation and a vulnerable target of free radical damage. Superoxide ($O_2^{\cdot-}$) generation from the ETC is converted to hydrogen peroxide (H_2O_2) via superoxide dismutase (SOD) enzymes. Notably, overexpression of Cu,Zn-SOD (SOD1); present in cytosol, lysosomes and mitochondrial intermembrane space (Okado-Matsumoto and Fridovich 2001), reduces tissue damage in adult cerebral stroke (Yang *et al.* 1994), but aggravates brain tissue damage after neonatal HI (Ditelberg *et al.* 1996). These effects of SOD1 overexpression are apparently due to the accumulation of H_2O_2 following HI, because of the limited expression of the H_2O_2 detoxifying enzymes (glutathione peroxidase and catalase) in the immature brain (Sheldon *et al.* 2007). While $O_2^{\cdot-}$ and H_2O_2 accumulation after HI likely contribute to the mitochondrial impairment observed in this study, there are other sources of ROS and reactive nitrogen species (RNS) that can impair mitochondrial function and increase protein carbonylation (Mueller-Burke *et al.* 2008).

Neuronal nitric oxide synthase (nNOS) is a site of RNS production known to be involved in HI (Black *et al.* 1995; Ferriero *et al.* 1996). Nitric oxide ($NO^{\cdot}$) production can have both detrimental and beneficial effects following injury. $NO^{\cdot}$ production is known to reversibly inhibit mitochondrial respiration at cytochrome c oxidase (complex IV) (Brown 2000). This inhibition may serve a regulatory function under physiological conditions; however,

excessive NO $\cdot$ production following HI could potentiate a reversal of ETC electron flow and exacerbate oxidative stress via O $_2\cdot$ leakage through complexes I and III. The combination of O $_2\cdot$ and NO $\cdot$ can form the highly toxic RNS, peroxynitrite (ONOO $^-$), which, in combination with H $_2$ O $_2$, can damage lipids, proteins and nucleic acids. Alternatively, NO $\cdot$ can also facilitate cerebral perfusion via vasodilation of the cerebrovasculature and inhibit apoptosis by impeding release of cytochrome c from the mitochondria (Blomgren and Hagberg 2006). Importantly, nNOS is the site of divergence in sexually dimorphic male and female cell death signaling cascades following HI, where female cell death proclivity involves cytochrome c release and caspase activation (Demarest and McCarthy 2015)., McCullough et al. (McCullough *et al.* 2005) demonstrated that the inhibition of nNOS following HI decreases lesion volume in male mice but actually increases lesion volume in females. Their findings point to a potentially beneficial role for nNOS in the female brain after HI (McCullough *et al.* 2005). This may be explained by nNOS inhibition of cytochrome c release or, as our results suggest, the possibility that ROS generation via mitochondria and/or nNOS might activate compensatory upregulation of antioxidant mechanisms in the female brain. Thus, inhibition of nNOS in the female brain following HI may exacerbate injury by abrogating compensatory upregulation of glutathione peroxidase and/or other antioxidant systems. Antioxidant enzyme activity of GPx is also higher in the cerebrospinal fluid of human neonates affected by HIE (Gulcan *et al.* 2005). Although the result was not statistically significant, it is worth noting that data from male and female newborns were not analyzed separately in this study by Gulcan et al. (Gulcan *et al.* 2005).

In the present study, we determined postnatal day 8 female rat pups had ~30% more brain GSH than males of the same age, and GSH in the female brain was bilaterally reduced to the levels of male shams after HI (Fig. 3a, b). This decrease in GSH coincided with an upregulation of total GPx activity in the ipsilateral hemisphere of females brain (Fig. 3f). The fact that there was no change in GPx activity in the contralateral hemisphere of females (Fig. 3d) likely reflects the ability of endogenous activities to handle the perturbations of hypoxia-alone. In contrast to the female response to HI, there was no change in GPx activity in total homogenate from male brain (Fig. 3 b). However, GPx activity in males was diminished specifically within the mitochondrial subcellular compartment of the contralateral hemisphere (Fig. 4b) and was paralleled by a decrease in phospholipid hydroperoxidase 4 (GPx4) protein (Fig. 4a). The neonatal brain is particularly susceptible to lipid peroxidation due to its high concentration of free iron and polyunsaturated fatty acids. Moreover, mitochondrial membranes contain a high concentration of polyunsaturated fatty acids (Vannucci and Hagberg 2004; Haynes *et al.* 2005). GPx4, a selenium-dependent phospholipid hydroperoxidase, is crucial for neuronal development and survival since global GPx4 knockout is embryonic lethal (Yant *et al.* 2003), and conditional knockout in adult animals causes neurodegeneration within 2 weeks (Yoo *et al.* 2012). The requirement of GPx4 for neuronal development and survival is likely because GPx4 is the only GPx isozyme which can effectively reverse lipid peroxidation (Ursini *et al.* 1986; Ursini *et al.* 1985). In combination with an increase in GSH in the contralateral hemisphere, and no change in the ipsilateral hemisphere in male brain after HI, the results from the present study suggest males may have a GPx deficiency relative to females. These differential responses to injury explain, at least in part, the higher degree of total protein oxidation (carbonyls) in

male brain than female brain after HI (Fig. 5–7). To the best of our knowledge, this is the first time protein carbonyl immunohistochemistry has been objectively quantified. Collectively, these results suggest that the female brain may possess an innate spare antioxidant capacity which contributes to the relative sparing of mitochondrial function. This may be an important factor in the sex differences observed in long term behavioral outcomes following HI.

Sex differences in behavioral outcome following the Rice-Vannucci HI model have been demonstrated in several studies (Waddell *et al.* 2015; Smith *et al.* 2014; Hill and Fitch 2012; Tsuji *et al.* 2010). Recently, a male specific deficit in novel object recognition task was found at 18–19 days following HI at postnatal day 10 rat pups (Waddell *et al.* 2015). Behavioral studies suggest that recognition memory is dependent on the perirhinal cortex, while location memory is hippocampus dependent during this task (Barker and Warburton 2011; Mendez *et al.* 2015). Our current observation that oxidative protein modifications significantly increase in both of these regions (Fig. 6, 7) suggest that sex dependent oxidative stress following HI may contribute to behavioral differences observed following neonatal HI injury. While our results are consistent with behavioral effects in other studies, including studies from our group (Waddell *et al.* 2015), we did not determine behavioral outcome in the current study. Future studies examining the time-course of oxidative stress and whether increases in protein carbonyl formation correlates with long-term behavioral outcome are needed to test whether these observations are interrelated.

As previously mentioned, ALCAR administration is neuroprotective in adult and neonatal brain injury. Xu *et al.* (2015) determined that ALCAR administration after HI prevented the decrease in GSH and prevented significant increases in lactate following HI (Xu *et al.* 2015). We previously observed that ALCAR reduces protein carbonyl formation in brain following a clinically relevant model of canine cardiac arrest (Liu *et al.* 1993). Consistent with these findings, the present study determined ALCAR administration following HI partially mitigated mitochondrial dysfunction (Fig. 1–2), prevented mitochondrial GPx impairment (Fig. 4b), and decreased protein carbonyl formation in the cortex (Fig. 5), perirhinal cortex (Fig. 6), and CA1 region of the hippocampus (Fig. 7). Notably, the impairment of antioxidant defense systems and increases in oxidative stress were only observed in male animals following HI and were prevented by ALCAR. These data suggest that ALCAR may act as an antioxidant following HI, either directly or via its ability to cause a reduced shift in cellular redox state (Zanelli *et al.* 2005). In addition to antioxidant properties, the acetyl group from ALCAR can be used for energy and as a precursor for the synthesis of glutamine, glutamate and GABA in astrocytes and neurons (Scafidi *et al.* 2010a). Our findings that ALCAR partially protected against mitochondrial dysfunction and prevented oxidative stress in the HI model taken together with demonstrated neuroprotection by ALCAR in a rat pediatric TBI model provide support for its use in pediatric neuroprotection clinical trials (Xu *et al.* 2015; Scafidi *et al.* 2010b).

Many studies have attributed the relative resilience of the female brain to ischemic injury to sex hormones (i.e. progesterone and estradiol) (Simpkins and Singh 2008; Singh *et al.* 2008). In the postnatal day 7–8 rat brain, estradiol, testosterone, and dihydrotestosterone levels in the brain are equivalent (Konkle and McCarthy 2011). Moreover, studies of primary

cultures of neurons from male and female brain found an increased susceptibility to oxidative stress in neurons derived from male brain (Du *et al.* 2004). Therefore, sex differences observed following HI are more likely due to hormone-independent mechanisms; however, the possibility of indirect hormonal epigenetic mechanisms, the influence of *in utero* hormone exposure, or differential downstream secondary messenger cascades cannot be excluded. Regardless, the results of the present study provide a possible mechanism which could contribute to the sex differences in long-term neurobehavioral outcomes observed clinically and in this experimental model of HIE (Smith *et al.* 2014; Hill and Fitch 2012). The current study provides the first evidence of sex differences in mitochondrial respiration and oxidative stress in neonatal HI; however, it is limited to 20–24 hours post injury. Additional studies examining the time course of sex dependent HI pathophysiology would be informative.

In summary, brain mitochondrial function was significantly more impaired in males than females following HI. The female brain exhibited higher levels of total brain glutathione in uninjured sham animals and was able to upregulate the GPx antioxidant system after injury. In contrast, we observed marked GPx antioxidant system impairment and protein carbonyl accumulation in the male brain following HI. These changes were attenuated by ALCAR treatment after HI in males. By parsing out the sex specific mechanisms of neonatal HI pathophysiology, we will gain invaluable insight into innate female neuroprotective/mitoprotective mechanisms which will aid in the future development of sex specific therapeutics for newborns suffering from HIE.

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Figure 1. Sex differences in complex I dependent mitochondrial respiratory impairment following HI

Representative traces for oxygen consumption by isolated brain mitochondria following sham (solid line), HI contralateral hemispheres (dashed line) and ipsilateral hemispheres (dotted line) from male (a) and female (b) rats. ADP-stimulated state 3 respiration from male (c) and female (d) rat brain. ATP synthase (complex V) inhibitor oligomycin was added to initiate state 4_O (resting proton leak) respiration in mitochondria from male (e) and female (f) rat brain. Proton ionophore (FCCP) mediated uncoupling of mitochondrial respiration in male (g) and female (h) brain. Respiratory control ratio for male (i) and female (j) brain

mitochondria. * $p < 0.05$, ** $p < 0.01$ vs. sham treated animals. # $p < 0.05$, ## $p < 0.01$ vs. opposite sex. Brackets denote significant hemisphere or treatment differences. $n = 4-6$ separate experiments.

Figure 2. Complex II dependent mitochondrial respiratory impairment following HI
ADP-stimulated state 3 respiration in mitochondria from male (a) and female (b) rat brain. ATP synthase (complex V) inhibitor Oligomycin was added to initiate state 4_O (resting proton leak) respiration in mitochondria from male (c) and female (d) rat brain. Proton ionophore (FCCP) mediated uncoupling of mitochondrial respiration in male (e) and female (f) derived brain mitochondria. Respiratory control ratio for male (g) and female (h) brain mitochondria *p<0.05, **p<0.01 vs. sham treated animals. # *p*<0.05 vs. opposite sex.

Brackets denote significant hemisphere or treatment differences. n=4–6 separate experiments.

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Figure 3. Sex differences in glutathione antioxidant defense capacity

Reduced glutathione (GSH) levels in total brain homogenates from male (a) and female (b) rat brain. Total glutathione peroxidase activity in brain homogenates from male (c,e) and female rats (d, f). * $p < 0.05$ vs. sham. # sex differences. (Sex $\times$ treatment) interaction $p = 0.006$. $n = 6$ /group.

Figure 4. Mitochondrial GPx impairment in males following HI

Sex dependent decrease in mitochondrial glutathione peroxidase 4 (GPx4) immunoreactivity (a) and impaired mitochondrial GPx activity in mitochondria isolated from male brain following HI (b). * $p < 0.05$ vs. sham. $n = 6$ /group.

Figure 5. Quantitative analysis of protein carbonyl immunohistochemistry in rat cortex following HI

Representative images of dinitrophenylhydrazine (DNPH)-derivitized protein carbonyl groups (green) and DAPI-stained cell bodies (blue) in cortical brain sections from male (left) and female (right) postnatal day 8 rat pups 24 hours after HI injury. Integrated intensity values reveal significant increases in contralateral and ipsilateral cortices from male brain (** $p < 0.001$) and significant reduction in carbonylation with ALCAR treatment after HI (** $p < 0.001$) in both hemispheres. (Sex $\times$ treatment) interaction $p < 0.001$. $n = 4/\text{group}$.

Figure 6. Quantitative analysis of protein carbonyl immunohistochemistry in the perirhinal cortex after HI

Representative images of DNPH-derivitized protein carbonyl groups (green) and DAPI-stained cell bodies (blue) in perirhinal cortex from male (left) and female (right) postnatal day 8 rats 24 hours after HI injury. Integrated intensity values reveal significant increases in contralateral and ipsilateral perirhinal cortices from male rats ($***p<0.001$) and significant reduction in carbonylation with ALCAR treatment ($***p<0.001$, $**p<0.01$) in both hemispheres. (Sex $\times$ treatment) interaction $p<0.001$. $n=4$ /group.

Figure 7. Quantitative analysis of protein carbonyl Immunohistochemistry in the CA1 region of the hippocampus after HI

Representative images of DNPH derivitized protein carbonyl groups (green) and DAPI-stained cell bodies (blue) in hippocampal brain sections from male (left) and female (right) postnatal day 8 rat pups 24 hours after HI injury. Integrated intensity values reveal significant increases in contralateral and ipsilateral cortices of brain from male rat pups (** $p < 0.001$) and significant reduction in carbonylation with ALCAR treatment

(*** $p < 0.001$, ** $p < 0.01$) in both hemispheres. (Sex $\times$ treatment) interaction $p < 0.001$. $n = 4$ /group.

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