

Mitochondrial dysfunction early after traumatic brain injury in immature rats

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Abstract

Mitochondria play central roles in acute brain injury; however, little is known about mitochondrial function following traumatic brain injury (TBI) to the immature brain. We hypothesized that TBI would cause mitochondrial dysfunction early (<4 h) after injury. Immature rats underwent controlled cortical impact (CCI) or sham injury to the left cortex, and mitochondria were isolated from both hemispheres at 1 and 4 h after TBI. Rates of phosphorylating (State 3) and resting (State 4) respiration were measured with and without bovine serum albumin. The respiratory control ratio was calculated (State 3/State 4). Rates of mitochondrial H₂O₂ production, pyruvate dehydrogenase complex enzyme activity, and cytochrome *c* content

were measured. Mitochondrial State 4 rates (ipsilateral/contralateral ratios) were higher after TBI at 1 h, which was reversed with bovine serum albumin. Four hours after TBI, pyruvate dehydrogenase complex activity and cytochrome *c* content (ipsilateral/contralateral ratios) were lower in TBI mitochondria. These data demonstrate abnormal mitochondrial function early (≤4 h) after TBI in the developing brain. Future studies directed at reversing mitochondrial abnormalities could guide neuroprotective interventions after pediatric TBI.

Keywords: brain mitochondria, cerebral metabolism, cytochrome *c*, development, oxidative stress.
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Millions of infants, children, and young adults are living with disabilities resulting from moderate to severe traumatic brain injury (TBI) in childhood or adolescence (Thurman *et al.* 1999). The emotional and economic burden of these severe brain injuries is significant. However, despite marked advances in the neurointensive care of these critically injured children, there are no neuroprotective treatments that have been shown to improve long-term neurologic outcome. Most components of bedside care are supportive and are directed at the control of intracranial hypertension and maintenance of adequate cerebral perfusion pressure (Adelson *et al.* 2003). In addition, many of the approaches are adapted from literature in adults and have not been adequately studied in pediatric populations. Development of effective neuroprotective strategies will require an improved understanding of the pathologic cascades operating following severe TBI in the developing brain. Investigation of age-dependent aspects of the subcellular response to injury will be necessary, as treatment strategies that have been effective in the adult brain may not be effective or could even be detrimental, in the immature brain (Pohl *et al.* 1999).

Mitochondria play a central role in cerebral energy metabolism, intracellular calcium homeostasis, and reactive oxygen species (ROS) generation and detoxification. Fol-

lowing TBI, the degree of mitochondrial injury or dysfunction can be an important determinant of cell survival or death (reviewed in Fiskum 2000). Many factors that both inhibit and promote apoptotic cell death work by influencing release of mitochondrial proteins, e.g. cytochrome *c*. In addition, post-traumatic pathways that promote necrotic cell death such as excitotoxicity and oxidative stress can have significant effects on mitochondrial metabolic functions. Growing evidence documents mitochondrial dysfunction after TBI in adult animal and human studies (Sullivan *et al.* 1999; Verweij *et al.* 2000; Lifshitz *et al.* 2003; Singh *et al.* 2006). A limited number of studies have described indirect evidence for mitochondrial dysfunction after pediatric TBI

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Abbreviations used: BSA, bovine serum albumin; CCI, controlled cortical impact; HSD, honestly significant difference; PBS, phosphate-buffered saline; PDHC, pyruvate dehydrogenase complex; PND, post-natal day; RCR, respiratory control ratio; ROS, reactive oxygen species; TBI, traumatic brain injury.

such as the presence of altered cerebral metabolism (Thomas *et al.* 2000) (Ashwal *et al.* 2000) and apoptotic cell death (Pohl *et al.* 1999). Animal studies suggest that brain mitochondria from the normal, developing brain are very different than those from mature animals [reviewed in (Robertson *et al.* 2006b) (Polster *et al.* 2003) (Holtzman and Moore 1973, 1975; Robertson *et al.* 2004)]. However, to our knowledge, no studies have directly evaluated mitochondrial bioenergetic activities after pediatric TBI. In the current study, we evaluated mitochondrial function after injury using a clinically relevant model of pediatric TBI in immature rats. We hypothesized that mitochondrial dysfunction occurs very early (≤ 4 h) after injury and that activities including oxidative phosphorylation, production of ROS, and release of cytochrome *c* are affected.

Materials and methods

Animals

This study was approved by the University of Maryland, Baltimore Animal Care and Use Committee. All care and handling of rats were in compliance with the National Institutes of Health guidelines. Immature (post-natal day (PND) 16–17), male Sprague–Dawley rats (Charles River Laboratories, Wilmington, MA, USA) weighing 32–40 g were used in all studies and were housed with littermates before surgery and after recovery from anesthesia.

TBI model

The injury paradigm for adult rats has been previously described (Robertson *et al.* 2006a) and was then modified for use in PND 16–17 rats for this study. Anesthesia was induced in a plexiglass chamber with 4% isoflurane. The head was then fixed in a stereotactic device and isoflurane (2–2.5%) with oxygen (30%) was administered via a nose-cone device for the duration of surgery. Rectal temperature was maintained at $37.0 \pm 0.5^\circ\text{C}$. A midline scalp incision was made, and a left parietal craniotomy was performed. A brain temperature probe was placed in the contralateral temporalis muscle with temperature maintained at $37.0 \pm 0.5^\circ\text{C}$. Rats were allowed a 30-min period of stable brain and rectal temperatures prior to TBI.

Traumatic brain injury was performed using the controlled cortical impact (CCI) device (Pittsburgh Precision Instruments, Pittsburgh, PA, USA) as previously described (Dixon *et al.* 1991; Robertson *et al.* 2006a). Injury was produced using a 6-mm metal impactor tip with a depth of penetration of 1.5 mm, a velocity of 5.5 ± 0.3 m/s, and a duration of deformation of 50 ms. Following injury, the bone flap was replaced, the craniotomy sealed, and the scalp incision was closed with interrupted sutures. At the completion of surgery, isoflurane was discontinued and rats were awakened and returned to their cages with littermates and mother. Sham rats underwent identical surgeries, with the exclusion of the CCI. Rats were not randomized, but treatment (TBI and sham) was alternated between animals on each experimental day of study.

Mitochondrial isolation

Mitochondrial isolation was performed 1 and 4 h after CCI. Forebrains were quickly removed and placed on an acrylic brain

matrice previously cooled in ice. A peritrauma segment of interest was rapidly dissected using razor blades placed 1 mm in front and 1 mm behind the area of injury on the matrice, for a total rostral to caudal length of approximately 8 mm. This segment of interest, therefore, contained the full section of impacted cortical tissue (that eventually becomes necrotic), a portion of peritrauma cortex (that did not experience direct injury), and deeper cortical structures such as the hippocampus and thalamus. The segments of interest were then separated into left (ipsilateral) and right (contralateral) samples and placed in ice-cold isolation buffer. Mitochondria (non-synaptosomal plus synaptosomal) were isolated from these samples as previously described (Starkov *et al.* 2004a; Robertson *et al.* 2006a) using digitonin to disrupt synaptosomal membranes. Isolated mitochondria were kept on ice for the duration of the experimental protocols.

Mitochondrial respiration

Mitochondrial oxygen consumption was measured after TBI using a Clark-type oxygen electrode (Hansatech Instruments/PP Systems, Amesbury, MA, USA) at 1 h ($n = 8$ TBI and $n = 6$ sham) and 4 h ($n = 5$ TBI and $n = 3$ sham) after injury. Mitochondrial samples from ipsilateral and contralateral hemispheres were measured separately. Assays were conducted at 37°C at a pH of 7.0 in a KCl medium (125 mmol/L KCl, 2 mmol/L KH_2PO_4 , 1 mmol/L MgCl_2 , and 20 mmol/L HEPES-KOH) in the absence and presence of defatted bovine serum albumin (BSA) at 1 mg/mL. The chamber was supplemented with 5 mmol/L pyruvate, 0.2 mmol/L malate, 1 $\mu\text{mol/L}$ EGTA, and 0.4 mmol/L ADP in a total volume of 0.5 mL. State 3 respiration was initiated by the addition of mitochondria (0.5 mg/mL), and State 4_0 respiration was induced by the addition of the ATP synthetase inhibitor oligomycin (2.5 $\mu\text{g/mL}$). State 4 respiration measured in the presence of oligomycin (State 4_0) is not equivalent to the traditional State 4 respiration measured after all ADP has been converted to ATP. However, for our measurements, we wanted to eliminate the contribution of ATP cycling via hydrolysis by contaminating ATPases and resynthesis by mitochondrial ATP synthetase. Thus, the oligomycin-induced State 4_0 rate of respiration reflects mitochondrial proton cycling limited by passive proton leakiness of the inner membrane. Mitochondrial respiratory energy coupling was evaluated by determining the respiratory control ratio (RCR), calculated as the ratio of the rate of ADP-stimulated State 3 respiration to the State 4_0 rate in the presence of oligomycin.

Mitochondrial ROS generation

Mitochondrial hydrogen peroxide (H_2O_2) production was measured 1 h after TBI using the fluorescent probe Amplex Red (Molecular Probes, Eugene, OR, USA) in five injured rats and four sham controls, as previously described (Starkov and Fiskum 2003; Schuh *et al.* 2005). Into the potassium chloride buffer described above (0.5 mL), the oxidizable substrates, pyruvate (5 mmol/L) and malate (0.2 mmol/L), and ADP (0.4 mmol/L) were added. Additional agents added were horseradish peroxidase (5 U/mL), Cu,Zn superoxide dismutase (20 U/mL), and 1 $\mu\text{mol/L}$ Amplex Red. Background measurements were taken for 2 min, and then mitochondria (0.25 mg/mL) were added to the cuvette during continuous fluorimetric recording. Two minutes later, oligomycin (2.5 $\mu\text{g/mL}$) was added followed by 1 $\mu\text{mol/L}$ rotenone. The slope of fluorescence increase was measured and baseline slope readings

subtracted to determine H₂O₂ production at 585 nm excitation and 550 nm emission wavelengths. The dye response was calibrated using a known amount of H₂O₂. This H₂O₂ concentration was calculated from light absorbance of the freshly prepared stock solution at 240 nm, using $E_{240} = 43.6 \text{ (mol/L)}^{-1} \text{ cm}^{-1}$.

Mitochondrial pyruvate dehydrogenase enzyme activity

Mitochondrial samples from 1 and 4 h after TBI were stored at -80°C , removed, and thawed on ice. Total pyruvate dehydrogenase complex (PDHC) activity was measured using methods previously described (Richards *et al.* 2006), adapted from previously published methods coupling the reduction of NAD to NADH with the reduction of a fluorescent dye (Hinman and Blass 1981). Briefly, freeze-thawed mitochondrial samples (40 $\mu\text{g/mL}$) were placed in a potassium phosphate buffer (50 mmol/L, pH 7.8; 37°C) with 0.5 mmol/L cocarboxylase, 5 mmol/L MgCl₂, 0.2 mmol/L coenzyme A, 5 mmol/L NAD, 2 U phosphotransacetylase, 0.3 U diaphorase, and 1 mmol/L resazurin. An inhibitor of lactate dehydrogenase, oxalate (0.5 mmol/L), was also added to minimize interference from lactate dehydrogenase (Elnageh and Gaitonde 1988). In these studies, the enzyme diaphorase reduces the dye resazurin to its fluorescent form, resorufin, which is fluorometrically measured at 585 nm excitation and 550 nm emission wavelengths. After obtaining baseline fluorescent readings, the reaction was started by the addition of 5 mmol/L pyruvate. The slope of fluorescence increase was measured and baseline slope readings subtracted to determine PDHC activity.

Mitochondrial cytochrome *c* content

Mitochondrial samples from 1 h ($n = 12/\text{group}$) and 4 h ($n = 6$ TBI and $n = 4$ sham) after TBI were assessed for cytochrome *c* content using an enzyme-linked immunosorbent assay (ELISA kit, R&D Systems, Minneapolis, MN, USA). Frozen mitochondrial samples (25 μg) were thawed and diluted in 100 μL of lysis buffer (0.5% nonidet P-40, 1% Triton X-100, 150 mmol/L NaCl, and 10 mmol/L Tris). These samples were further diluted (1 : 60) in calibration diluent and loaded into wells for a final concentration of 1.66 μg protein/mL. Mitochondrial cytochrome *c* concentration was determined according to the manufacturer's ELISA protocol.

Hippocampal cell counts

At 7 days after TBI, rats ($n = 16$ TBI and $n = 10$ sham) were anesthetized using an overdose of ketamine (200 mg/kg, ip) and transcardially perfused with saline, followed by fixation in paraformaldehyde (4%). Brains were removed and post-fixed in 4% paraformaldehyde for 2 h at 4°C . They were then placed in 30% sucrose solution until they sunk ($\sim 3\text{--}4$ days). This resulted in comparable amounts of shrinkage among all of the animals studied. Brains were sectioned coronally (40 μm) using a cryostat and were placed into antifreeze cryoprotectant solution for storage at -20°C in a 1 : 6 series. For analysis of neuronal cell counts using NeuN labeling, a standard free-floating immunocytochemistry protocol was used as previously described (Hoffman *et al.* 2001). Briefly, sections were rinsed thoroughly to remove cryoprotectant with phosphate-buffered saline (PBS). Sections were incubated with the primary antibody, mouse monoclonal anti-NeuN (1 : 100 000; Chemicon, Temecula, CA, USA), in PBS with 0.4% Triton-X for 48 h at 4°C . Sections were then rinsed with PBS and incubated with

the secondary antibody, biotinylated anti-mouse antibody (Vector Laboratories, Burlingame, CA, USA) (1 : 500) in PBS with 0.4% Triton-X for 1 h. After rinsing again in PBS, slices were prepared with the VectorStain Elite ABC kit (Vector Laboratories). Following a series of rinses with PBS and sodium acetate (0.175 mol/L), the slices were then placed into a Ni-diaminobenzadine H₂O₂ chromogen solution (250 mg Ni sulfate, 2 mg diaminobenzadine, and 8.3 μL 3% H₂O₂/10 mL 0.175 sodium acetate solution). Staining was terminated by transferring to the sodium acetate solution. Stained slices were then mounted on slides for quantification.

Quantification of hippocampal neuronal cells was performed as previously described (Robertson *et al.* 2006a). Analysis utilized a computer-assisted image analysis system consisting of a Nikon Eclipse 800 photomicroscope, a Retiga EX digital camera (Biovision Technologies, Exton, PA, USA), and a Macintosh G4 computer with IP Spectrum software (Scientific Image Processing, Scanalytics, Fairfax, VA, USA). Using 40x magnification, an equivalent slice through the hippocampus in the CA1 and CA3 subfields from both ipsilateral and contralateral hemispheres was analyzed. In order to provide consistency, the section selected for analysis was the section of hippocampus directly beneath the central area of cortical injury in each rat. In each analyzed subfield, the numbers of normally stained NeuN neurons were counted. For this study, neurons with more intense nuclear reactivity relative to the cytoplasm were counted as 'normal' as described in (Robertson *et al.* 2006a). Two 40x fields were analyzed per hippocampal subfield on both sides in every rat. Data are expressed as the number of 'normal' neurons per high-powered field.

Cortical tissue loss analysis

Quantification of tissue loss was performed on separate sets of rats, comparing injured ($n = 8$) to sham ($n = 5$) at 7 days after CCI. This time point was selected to allow for meaningful comparison of tissue loss between our model in immature rats and other TBI studies. Rats were anesthetized and perfused as described for hippocampal cell counts. Serial coronal sections were taken at 1 mm intervals rostral to caudal through the whole brain. Sections were mounted on slides and stained with cresyl violet. Using Image J software (NIH, Bethesda, MD, USA), the margins of the contusion and left (ipsilateral) and right (contralateral) hemispheres were outlined. Area of interest (mm^2) was calculated by conversion from measurements of photomicrographs of a ruler (pixels/mm) processed in the same method as slides. Standard calculation of volume (area \times distance between slides) was performed. Volume of left hemisphere, right hemisphere, and contusion were recorded. Data are expressed as contusion volume (mm^3) and percentage tissue loss using the formula: $100 - [(\text{left/ipsilateral cortex volume} + \text{right/contralateral cortex volume}) \times 100]$.

Statistical analysis

All data are presented as mean \pm SEM. The ipsi/contra ratios of mitochondrial respiration, PDHC activity, and cytochrome *c* content were transformed to the square root to provide a more Gaussian distribution. The square root of the ipsi/contra ratios of TBI and sham controls were then compared across time (1 and 4 h) by one-way analysis of variance (ANOVA) with *post hoc* individual two-way comparisons made using Tukey's honestly significant difference (HSD). Mitochondrial ROS production and hippocampal cell counts

were compared using ANOVA across groups at a single time point, with Tukey's HSD *post hoc* individual comparisons.

Results

Mitochondrial respiration

We compared the ipsi/contra ratio of State 3, State 4₀, and RCR values across groups (TBI and sham) and time (1 and 4 h). The State 3 ipsi/contra ratios were significantly different ($p < 0.05$ by ANOVA on transformed data), with a trend toward lower State 3 ratio at 4 h in TBI (0.83 ± 0.06) compared with sham (1.10 ± 0.06 , $p = 0.08$ by Tukey's HSD *post hoc* analysis). The State 4₀ ipsi/contra ratios were also significantly different ($p < 0.05$ by ANOVA on transformed data), with a significant increase in State 4₀ ratio at 1 h in TBI (1.36 ± 0.16) compared with sham (0.89 ± 0.07 by Tukey's HSD). The RCR ipsi/contra ratios were not significantly different across time between TBI (1 h = 0.82 ± 0.09 ; 4 h = 0.96 ± 0.05) and sham (1 h = 0.99 ± 0.05 ; 4 h = 1.02 ± 0.08).

To test the hypothesis that increases in resting respiration (State 4₀) are due to the presence of free fatty acids, a comparison was made between mitochondrial oxygen consumption in the absence and presence of defatted BSA, which is known to absorb free fatty acids. In the presence of BSA, State 3 respiratory rates remained generally unchanged and State 4₀ rates were lower with greater absolute RCR values in the presence of BSA compared with measurements without BSA (Table 1). We compared ipsi/contra ratios of State 3, State 4₀, and RCR values with and without BSA (Fig. 1). State 3 ratios were not different across groups (Fig. 1a). The State 4₀ ratio was higher after TBI in the absence of BSA compared with TBI in the presence of BSA and both sham groups ($p < 0.05$ by ANOVA on transformed data; Fig. 1b). The RCR ipsi/contra ratio was greater in TBI at 4 h compared with TBI at 1 h ($p < 0.05$ by ANOVA on transformed data; Fig. 1c).

Mitochondrial ROS generation

As mitochondria may be one important source of ROS that contribute to tissue injury after TBI (Robertson *et al.* 2006b) measurements were made of mitochondrial ROS formation

in vitro under conditions similar to those used for measurements of respiration. Overall, the absolute amount of H₂O₂ generated by isolated mitochondria from TBI rats was lower than the amount generated by mitochondrial from sham rats in all conditions tested (Fig. 2). However, there was not statistical significance in this difference. The most pronounced differences were apparent when ROS was measured in the presence of the Complex I inhibitor, rotenone ($p = 0.05$ by ANOVA comparing all means), which provides the maximal rate of ROS production by sites present within or proximal to this component of the electron transport chain (Starkov *et al.* 2004a). There were no significant differences in the rates of ROS generations when comparing ipsilateral (injured) to contralateral (uninjured) samples in either TBI or sham rats.

Mitochondrial pyruvate dehydrogenase enzyme activity

Values for total PDHC activity (10–63 nmol/min/mg mitochondrial protein) were consistent with values previously reported (Jope and Blass 1975; Hinman and Blass 1981). We compared the ipsi/contra ratio of PDHC activity across groups (TBI and sham) and time (1 and 4 h; Fig. 3). The PDHC activity ratio was significantly different ($p < 0.05$ by ANOVA on transformed data), with a lower activity after TBI versus sham at 4 h ($p < 0.05$ by Tukey's HSD; Fig. 3). The PDHC activity ratios were not different between TBI and sham at 1 h.

Mitochondrial cytochrome *c* content

The level of cytochrome *c* immunoreactivity present in isolated brain mitochondria was determined as redistribution of cytochrome *c* from the mitochondrial intermembrane space to the cytosol is a common trigger of caspase-dependent apoptosis and occurs in several models of adult TBI (Buki *et al.* 2000; Lewen *et al.* 2001; Sullivan *et al.* 2002). At 1 h after TBI, mitochondrial samples from injured rats had ~10% lower measurable cytochrome *c* content (ipsi = 8973 ± 673 and contra = 9113 ± 616 ng/mg mitochondrial protein) than samples from sham-operated rats (ipsi = $10\,696 \pm 600$ and contra = $10\,096 \pm 418$ ng/mg). At 4 h after TBI, the cytochrome *c* content in mitochondria isolated from the side ipsilateral to injury (7579 ± 564 ng/mg) was ~20%

Table 1 Mitochondrial respiratory measurements +/- BSA at 1h after CCI

		State 3 (nmol/min/mg)		State 4 ₀ (nmol/min/mg)		RCR State 3/State 4 ₀	
		<i>Ipsi</i>	<i>Contra</i>	<i>Ipsi</i>	<i>Contra</i>	<i>Ipsi</i>	<i>Contra</i>
- BSA	TBI	58.0 ± 6.1	60.1 ± 8.4	8.4 ± 0.6	6.7 ± 0.8	7.0 ± 0.5	8.9 ± 0.8
	Sham	56.2 ± 5.3	65.9 ± 6.5	6.3 ± 0.6	7.4 ± 0.9	9.5 ± 1.3	9.7 ± 1.4
+ BSA	TBI	62.1 ± 6.8	61.4 ± 7.0	4.7 ± 0.5	5.2 ± 0.6	13.5 ± 0.7	12.0 ± 1.1
	Sham	62.7 ± 5.3	74.6 ± 6.5	5.4 ± 0.4	5.8 ± 0.4	11.9 ± 1.1	13.0 ± 0.8

RCR, respiratory control ratio; BSA, bovine serum albumin; *Ipsi*, ipsilateral; *Contra*, contralateral; TBI, traumatic brain injury.

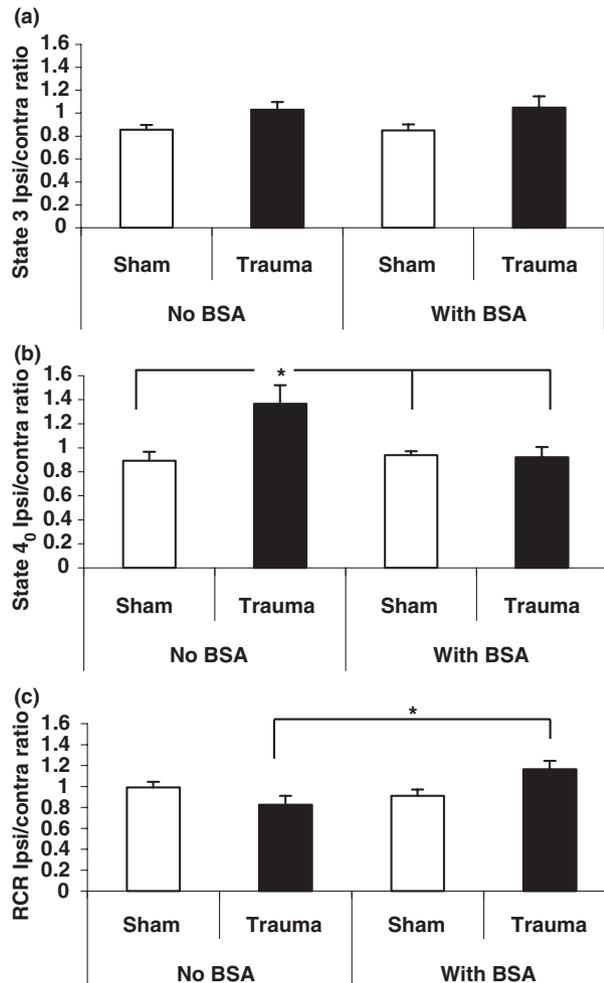


Fig. 1 Ipsilateral to contralateral ratios of State 3 (a), State 4₀ (b) rates, and respiratory control ratio (RCR) (c) in the absence and presence bovine serum albumin (BSA) (1 mg/mL), comparing mitochondrial ratios from sham (open bars) to traumatic brain injury (TBI; closed bars) at 1 h. Isolated mitochondria were incubated at 37°C with 5 mmol/L pyruvate and 0.2 mmol/L malate in a KCl medium. State 3 respiration was in the presence of 0.4 mmol/L ADP and State 4₀ was induced by the addition of oligomycin. The RCR was determined as a ratio of State 3 to State 4₀ rates. State 3 ratios were not different between groups (1a). Mitochondria isolated from TBI rats had an increased State 4₀ ipsi/contra ratio compared with both sham groups and TBI with BSA (b; * $p < 0.05$ one-way ANOVA with *post hoc* Tukey honestly significant difference on transformed data). RCR ipsi/contra ratio from TBI rats was the same as that from sham rats in the absence of BSA (c; $p = \text{NS}$ on transformed data). With BSA, the RCR ipsi/contra ratio was greater in TBI rats versus TBI without BSA (c; * $p < 0.05$ one-way ANOVA with *post hoc* Tukey honestly significant difference on transformed data).

less than the content from the contralateral hemisphere (9531 ± 374 ng/mg). We compared the ipsi/contra ratio of cytochrome *c* content across groups (TBI and sham) and time (1 and 4 h; Fig. 4). The cytochrome *c* content ratio was significantly different ($p < 0.05$ by ANOVA on transformed

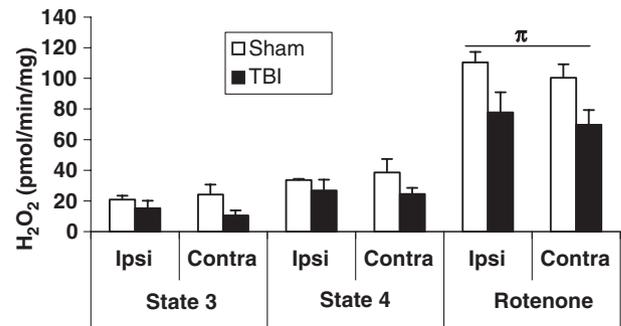


Fig. 2 Fluorimetric measurements of H₂O₂ production in isolated rat brain mitochondria, incubated at 37°C with 5 mmol/L pyruvate and 0.2 mmol/L malate in a KCl medium, with horseradish peroxidase (5 U/mL), Cu,Zn superoxide dismutase (20 U/mL), and 1 μmol/L Amplex Red. State 3 rate was in the presence of 0.4 mmol/L ADP and State 4 rate was induced by the addition of oligomycin. Rates were then measured in the presence of the Complex I inhibitor, rotenone. The rate of H₂O₂ production was the same across groups in State 3 and State 4 conditions. There was a trend toward difference in the rate of H₂O₂ production between groups in the presence of rotenone (π $p = 0.05$ by ANOVA).

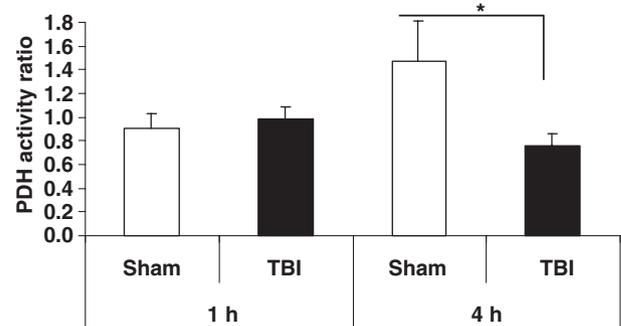


Fig. 3 The ipsi/contra ratio of pyruvate dehydrogenase complex (PDHC) maximal enzyme activity measured spectrofluorimetrically in isolated mitochondria at 1 and 4 h after sham (open bars) or traumatic brain injury (TBI; closed bars) in a potassium phosphate buffer at 37°C. Mitochondria from TBI rats had a lower ipsi/contra ratio of PDHC activity than sham rats at 4 h (* $p < 0.05$ one-way ANOVA with *post hoc* Tukey honestly significant difference on transformed data).

data), with a lower content after TBI at 4 h versus both sham groups ($p < 0.05$ by Tukey's HSD; Fig. 4). The cytochrome *c* content ratio after TBI at 1 h was not significantly different from sham.

Hippocampal cell counts and cortical tissue loss

In order to correlate early mitochondrial functional abnormalities with severity of injury in our TBI model, we quantified the histologic injury using hippocampal neuronal cell counts and cortical tissue loss analysis at 7 days after TBI. Representative examples of high-power photomicrographs from the CA1 and CA3 subfields of hippocampus in

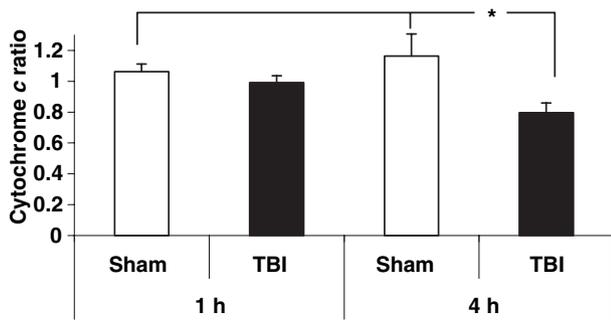


Fig. 4 The ipsi/contra ratio of mitochondrial cytochrome *c* content measured by ELISA in isolated mitochondria at 1 and 4 h after sham (open bars) or traumatic brain injury (TBI; closed bars). Mitochondria from TBI rats at 4 h after injury had a lower ipsi/contra ratio of cytochrome *c* content compared with both sham groups (* $p < 0.05$ one-way ANOVA with *post hoc* Tukey honestly significant difference on transformed data).

injured versus sham rats after TBI are seen in Fig. 5. TBI resulted in significant neuronal cell injury in the ipsilateral hippocampus, with ~50% reduction in normal neurons in both the CA1 (Fig. 6a) and CA3 (Fig. 6b) subfields at 7 days after TBI ($p < 0.001$ TBI vs. Sham). Overall, TBI resulted in a contusion volume of $32.9 \pm 4.2 \text{ mm}^3$, which corresponded to a $9.8 \pm 1.6\%$ hemispheric volume loss.

Discussion

In a clinically relevant animal model of pediatric TBI, we demonstrate evidence for very early (≤ 4 h) mitochondrial dysfunction on the side ipsilateral to injury. Mitochondrial functional abnormalities include alterations in mitochondrial

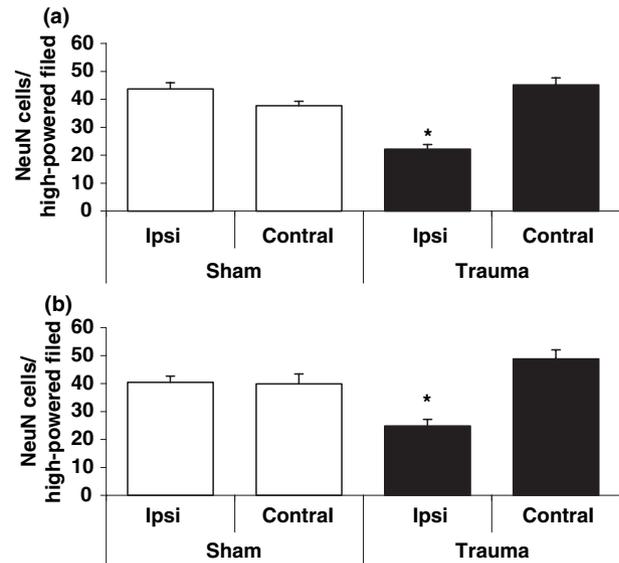


Fig. 6 Traumatic brain injury reduced hippocampal neuronal cells at 7 days after injury. Neuronal cell counts (normal NeuN/high-powered field) are reduced on the ipsilateral (injured) side compared with the uninjured side and sham neuronal cell counts (* $p < 0.001$) in both CA1 (a) and CA3 (b) subfields of the hippocampus.

respiration, a strong trend toward reduced H_2O_2 production in the presence of rotenone, and reduced activity of the metabolic enzyme PDHC. In addition, there was evidence for loss of mitochondrial cytochrome *c* content by 4 h after TBI. To our knowledge, this is the first study to directly evaluate mitochondrial function after TBI in the developing brain.

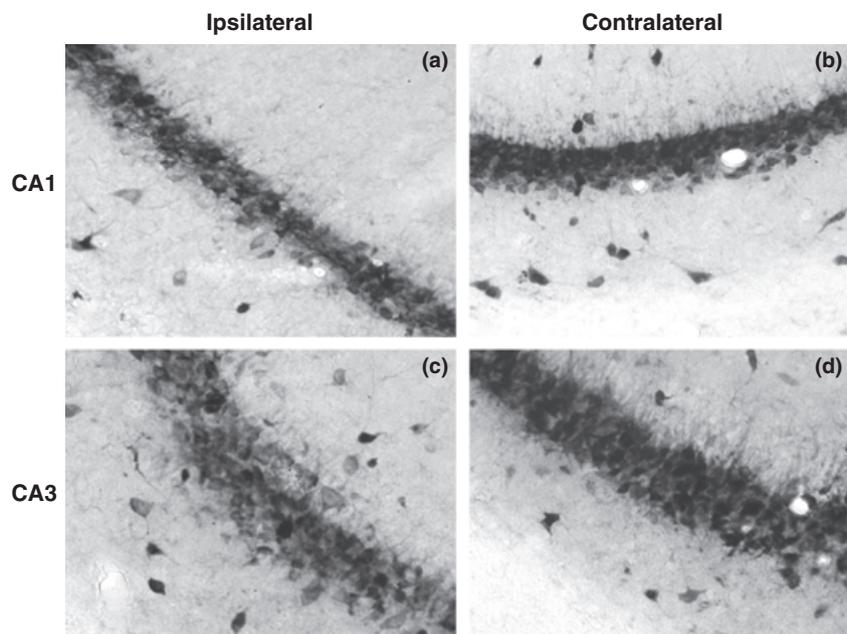


Fig. 5 Representative photomicrographs of the CA1 (a and b) and CA3 (c and d) subfields of the hippocampus stained for NeuN from ipsilateral (a and c) and contralateral (b and d) hemispheres at 7 days after traumatic brain injury. The ipsilateral CA1 and CA3 subfields show diffuse loss of total hippocampal neurons, with many abnormally stained neurons and few normally stained neurons among remaining cells. The contralateral side shows preservation of normal NeuN-stained neurons.

We selected early (≤ 4 h), pre-lethal time points for measuring mitochondrial function to minimize contribution from dead tissue. Mitochondria were isolated from a range of tissue types, including the complete area of cortical impact, regions of peritrauma cortex, and subcortical structures. As a result of this heterogeneity of mitochondrial sampling, it is possible that the degree of mitochondrial dysfunction *in vivo* could be underestimated by our measurements.

Alterations in mitochondrial respiratory capacity have been demonstrated following TBI in adult animals and humans (Verweij *et al.* 1997, 2000; Xiong *et al.* 1997). Studies in the CCI model of TBI defined significant alterations in mitochondrial respiration that began early (< 1 h) and persisted (14 day) (Xiong *et al.* 1997). These investigators found reductions in RCRs in both hemispheres, with ipsilateral impairments in State 3 respiration and contralateral increases in State 4 rates. Similar to these investigations, we documented early (≤ 4 h) abnormalities in mitochondrial respiration with immediate (≤ 1 h) increases in State 4 rates, followed by a trend in reduction of State 3 respiration by 4 h after TBI.

Elevation of State 4 respiratory rates and uncoupling of mitochondrial respiration indicate a loss of mitochondrial membrane integrity. One possible cause of this is elevated inner membrane proton permeability due to the presence of free fatty acids (Shinohara *et al.* 1995). The reversibility with BSA suggests a role for free fatty acids, as albumin is an avid binder of fatty acids and was shown previously to reverse increases in State 4 respiration (Hillered and Chan 1988). Despite the fact that BSA was present in our mitochondrial isolation medium, its presence in the respiratory medium reversed the TBI-induced elevation of State 4 respiration. This observation suggests that a neurochemical alteration occurs *in vivo* that promotes the production of free fatty acids *in vitro* when the ice-cold mitochondrial stock suspension is diluted into respiratory medium maintained at 37°C.

One possible explanation for the BSA-sensitive elevation of State 4 respiration is oxidation of phospholipids fatty acyl groups, which is known to greatly accelerate their hydrolysis by phospholipases (Sevanian *et al.* 1988). TBI in both animals and humans has been shown to result in early, marked increases in brain free radicals and activation of phospholipases (Lewen *et al.* 2000; Tyurin *et al.* 2000) (Varma *et al.* 2003), (Bayir *et al.* 2002, 2004) with subsequent elevations in brain free fatty acids (Phillis and O'Regan 2003), as early as 1–5 min after TBI in adult rats and mice (Hall *et al.* 1993; Lyeth *et al.* 1996). A recent study in adult TBI showed that the early (< 6 h) reduction in RCR was a result of elevation in State 4 rates and paralleled the appearance of markers of mitochondrial oxidative injury such as 4-hydroxynonenal, a marker of lipid peroxidation (Singh *et al.* 2006). We have previously demonstrated similar uncoupling of mitochondrial respiration with increases in State 4 rates after TBI (Robertson *et al.* 2006a), with

reversal by progesterone, potentially through antioxidant and membrane stabilization properties.

A recent series of studies by Kagan *et al.* has identified the mitochondrial phospholipid, cardiolipin, as a major target of oxidative injury (Kagan *et al.* 2005, 2006; Bayir *et al.* 2006). These investigators have described a unique interaction between cardiolipin and the oxidized form of cytochrome *c*, forming a complex that is a potent peroxidase for cardiolipin. They suggest that this oxidized cardiolipin shows minimal affinity to cytochrome *c*, facilitating its release (Ott *et al.* 2002; Petrosillo *et al.* 2003). Our finding that ipsilateral hemisphere mitochondria exhibit significantly lower cytochrome *c* content at 4 h post-TBI may therefore be linked to the same oxidative stress mechanism that could be responsible for elevated State 4 respiration. Studies in adult TBI (Buki *et al.* 2000; Lewen *et al.* 2001; Sullivan *et al.* 2002) and immature hypoxic-ischemic brain injury (Hagberg 2004; Vannucci and Hagberg 2004) (Nakajima *et al.* 2000; Yakovlev *et al.* 2001) have also documented the release of cytochrome *c* from mitochondria, occurring as early as 30 min to 1 h after injury. One study (Zhu *et al.* 2005) showed that cytochrome *c* release was greatest in the youngest mice (PND 5 and 9) compared with juvenile (PND 21) and adult (PND 60) mice. Studies in pediatric TBI are limited, but one study described detectable cytochrome *c* in the cerebrospinal fluid content of infants and children following severe TBI (Satchell *et al.* 2005). Our study found that the mitochondrial release of cytochrome *c* begins early (< 4 h) after TBI in the immature rat. Aside from functional alterations in mitochondrial bioenergetics, it is possible that this degree of cytochrome *c* release could promote significant apoptotic cell death without detectable effects on State 3 respiration. This has potentially important implications when considering neuroprotective strategies for infants and children after TBI.

An unexpected finding of our studies was a trend toward a reduction in rotenone-stimulated H₂O₂ production by mitochondria isolated from injured rats. This suggests that the flow of electrons into or through Complex I of the electron transport chain may have been reduced after injury. This could be the result of alterations in the rate of one or more oxidation/reduction reactions that occur within Complex I (Allen *et al.* 1995), lower levels of electron donation from NADH due to catabolism of mitochondrial pyridine nucleotides (Du *et al.* 2003), or from lower levels of reducing equivalents passed to NAD⁺ from metabolism of NAD-linked substrates, e.g. pyruvate. The finding that both pyruvate and α -ketoglutarate dehydrogenases are significant direct sources of ROS (Starkov *et al.* 2004b; Tretter and Adam-Vizi 2004) suggest that a reduction in the activity of one of these enzymes could explain impaired mitochondrial H₂O₂ production with pyruvate plus malate as substrates, even in the presence of normal State 3 respiration. While a modest reduction in mitochondrial H₂O₂ production was

observed in our *in vitro* measurements, this observation does not rule out an important contribution of mitochondrial free radical production to oxidative stress *in situ*, where conditions, e.g. ambient concentrations of nitric oxide, could impair respiration in ways that would promote mitochondrial ROS generation (Moncada and Bolanos 2006).

We did not directly evaluate all potential contributors to mitochondrial ROS production in this study, but we did measure the activity of one key metabolic enzyme, the PDHC. PDHC is the important bridge between anaerobic and aerobic metabolism and is one of several metabolic enzymes that are very sensitive to oxidative injury (Fiskum *et al.* 2004; Martin *et al.* 2005). Its activity is reduced following cerebral ischemia/reperfusion (Cardell *et al.* 1989; Zaidan and Sims 1993, 1997; Bogaert *et al.* 1994, 2000; Zaidan *et al.* 1998), and reperfusion-dependent inactivation is spared by conditions that minimize oxidative stress (Richards *et al.* 2006). To our knowledge, no one has directly evaluated PDHC activity after injury to the developing brain. This may be especially important, as the PDHC enzyme content and activity increase throughout post-natal development (Booth *et al.* 1980; Malloch *et al.* 1986; Clark *et al.* 1993; Cullingford *et al.* 1994), potentially leading to greater effects on cerebral metabolism than a comparable reduction in a mature brain.

In summary, we demonstrate that alterations in the function of brain mitochondria begin within 4 h of severe TBI in immature rats. This could provide improved understanding of the subcellular response to injury in the young brain and could ultimately guide the development of neuroprotective interventions for critically injured children. Future studies will be needed to evaluate their relationship to cell death and cognitive outcome. In addition, experiments should evaluate mitochondrial function at other developmental ages (e.g. PND < 10 and PND 20–30).

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