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TIME COURSES OF POST-INJURY MITOCHONDRIAL OXIDATIVE DAMAGE AND RESPIRATORY DYSFUNCTION AND NEURONAL CYTOSKELETAL DEGRADATION IN A RAT MODEL OF FOCAL TRAUMATIC BRAIN INJURY

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Abstract

Traumatic brain injury (TBI) results in rapid reactive oxygen species (ROS) production and oxidative damage to essential brain cellular components leading to neuronal dysfunction and cell death. It is increasingly appreciated that a major player in TBI-induced oxidative damage is the reactive nitrogen species (RNS) peroxynitrite (PN) which is produced in large part in injured brain mitochondria. Once formed, PN decomposes into highly reactive free radicals that trigger membrane lipid peroxidation (LP) of polyunsaturated fatty acids (e.g. arachidonic acid) and protein nitration (3-nitrotyrosine, 3-NT) in mitochondria and other cellular membranes causing various functional impairments to mitochondrial oxidative phosphorylation and calcium (Ca^{2+}) buffering capacity. The LP also results in the formation of neurotoxic reactive aldehyde byproducts including 4-hydroxynonenal (4-HNE) and propenal (acrolein) which exacerbates ROS/RNS production and oxidative protein damage in the injured brain. Ultimately, this results in intracellular Ca^{2+} overload that activates proteolytic degradation of α -spectrin, a neuronal cytoskeletal protein. Therefore, the aim of this study was to establish the temporal evolution of mitochondrial dysfunction, oxidative damage and cytoskeletal degradation in the brain following a severe controlled cortical impact (CCI) TBI in young male adult rats. In mitochondria isolated from an 8mm diameter cortical punch including the 5 mm wide impact site and their respiratory function studied *ex vivo*, we observed an initial decrease in complex I and II mitochondrial bioenergetics within 3 hours (h). For complex I bioenergetics, this partially recovered by 12–16h, whereas for complex II respiration the recovery was complete by 12h. During the first 24h, there was no evidence of an injury-induced increase in LP or protein nitration in mitochondrial or

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cellular homogenates. However, beginning at 24h, there was a gradual secondary decline in complex I and II respiration that peaked at 72h. post-TBI that coincided with progressive peroxidation of mitochondrial and cellular lipids, protein nitration and protein modification by 4-HNE and acrolein. The oxidative damage and respiratory failure paralleled an increase in Ca^{2+} -induced proteolytic degradation of the neuronal cytoskeletal protein α -spectrin indicating a failure of intracellular Ca^{2+} homeostasis. These findings of a surprisingly delayed peak in secondary injury, suggest that the therapeutic window and needed treatment duration for certain antioxidant treatment strategies following CCI-TBI in rodents may be longer than previously believed.

TOC Image

Keywords

traumatic brain injury; mitochondria; peroxynitrite 4-hydroxynonenal; acrolein; calpain

Introduction

Mitochondria serve a crucial role in cellular bioenergetics, function and survival; and yet, there is overwhelming evidence of their involvement in oxidative toxicity and ensuing neuropathology following traumatic brain injury (TBI) (Bains and Hall, 2012; Facecchia et al., 2011; Fiskum, 2000; Hall et al., 2010; Nicholls and Budd, 2000; Robertson, 2004). Mechanisms of secondary injury include the generation of reactive oxygen and nitrogen species (ROS and RNS, respectively) and intracellular Ca^{++} dysregulation which ultimately lead to cellular dysfunction and neurodegeneration. Mitochondria not only initiate the production of these ROS/RNS, but enhance cellular and mitochondrial dysfunction by becoming targets of their own damaging products through a vicious cycle of oxidative toxicity (Radi et al., 2002b). The increased production of free radicals leads to oxidative damage to essential cellular components including neurons, astrocytes and vascular elements (Hall et al., 2010).

TBI causes an increase in mitochondrial Ca^{2+} uptake which stresses the mitochondria and causes activation of mitochondrial Ca^{2+} -activated nitric oxide synthase (NOS) which leads to the mitochondrial production of nitric oxide ($\text{NO}^\bullet$), as well as leakage of superoxide radical ($\text{O}_2^{\bullet-}$). These two free radicals rapidly react with each other to form peroxynitrite

(PN) anion (ONOO^-) (Bains and Hall, 2012; Hall et al., 2010). At physiological pH, a large amount the PN anion picks up a proton (H^+) to form peroxynitrous acid (ONOOH) which will then decompose to form the highly reactive nitrogen dioxide ($\text{NO}_2^\bullet$) and hydroxyl ($\bullet\text{OH}$) radicals. Alternatively, the PN anion can react with carbon dioxide (CO_2) to form nitrosoperoxocarbonate (ONOOCO_2^-) which will likewise decompose to form $\text{NO}_2^\bullet$ and carbonate ($\text{CO}_3^{\bullet-}$) radicals (Radi et al., 2001). Each of these radicals will trigger lipid peroxidation (LP)-mediated oxidative damage to the mitochondrial LP- susceptible membrane polyunsaturated fatty acids (e.g. arachidonic acid, docosahexaenoic acid, linoleic acid) and nitration of proteins (Hall et al., 2010; Radi, 2004; Radi et al., 1991; Radi et al., 2002a). As this oxidative damage progresses within the mitochondrion, it causes loss of mitochondrial membrane potential, respiratory failure that compromises ATP production and ultimately mitochondrial permeability transition (mPT). When this occurs, the Ca^{2+} that was taken up by the mitochondrion soon after TBI is released back into the cytoplasm where it will exacerbate the activation of the Ca^{2+} -activated cysteine proteases calpain and caspase 3 causing proteolysis of neuronal cytoskeletal proteins such as α -spectrin as well as other substrates (Hall et al., 2010). Moreover, since each of the PN forms has a rather long half-life (~1 second) this provides them with a diffusion radius sufficient for them to leave the mitochondria before breaking down into their highly reactive radicals, enabling mitochondrially-generated PN to damage other cellular components (e.g. endoplasmic reticulum, plasma membrane) (Radi, 2004).

Brain tissue is highly affected by LP-mediated membrane damage because of the higher content of peroxidation-susceptible polyunsaturated fatty acids in neural membrane lipids in comparison with other tissues (Hall et al., 2010; Nobre Jr. et al., 2009). In addition to LP producing membrane phospholipid disruption the peroxidized lipids ultimately break down to form the neurotoxic reactive aldehydes such as 4-hydroxy-2-nonenal (4-HNE) and propenal (acrolein) (Halliwell and Gutteridge, 2008). The 4-HNE and acrolein react with proteins, DNA/RNA and phospholipids, further compromising mitochondrial and cellular structural and functional integrity (Esterbauer et al., 1991; Kehrer and Biswal, 2000; Peterson and Doorn, 2004). Previous studies have demonstrated the involvement of 4-HNE (Deng et al., 2007; Mbye et al., 2009; Mustafa et al., 2010a) and acrolein (Hamann et al., 2008; Shi et al., 2011)-mediated damage in post-traumatic neuropathology. In addition, nitration of tyrosine residues by $\text{NO}_2^\bullet$ leads to an accumulation of 3-nitrotyrosine (3-NT), a known biomarker of PN action in TBI models (Bains and Hall, 2012; Singh et al., 2006a).

Disruption of mitochondrial Ca^{2+} homeostasis resulting from impaired oxidative phosphorylation and Ca^{2+} buffering (Xiong et al., 1997b) has been shown to exacerbate neuronal Ca^{2+} overload and the activation of Ca^{2+} -activated cysteine proteases including calpain and caspase 3. Post-traumatic activation of calpain mediates the proteolytic degradation of α -spectrin, a neuronal cytoskeletal protein into two α -spectrin breakdown products (SBDPs) of 150 kD and 145 kD (Saatman et al., 1996a; Siman et al., 1984; Siman et al., 1989; Wang, 2000b). Caspase 3 activation, reflective of apoptotic neurodegeneration, further produces a slightly different 150 kD SBDP from which a caspase 3-specific 120 kD SBDP is generated. These diagnostic TBI-related biomarkers have been employed by multiple laboratories conducting preclinical TBI studies (Hall et al., 2005; Kupina et al., 2002; Ringger et al., 2004; Saatman et al., 1996b; Wang, 2000a; Yan and Jeromin, 2012).

It has been well established that progressive mitochondrial dysfunction and ultrastructural damage occurs in the injured brain, beginning within the first post-injury h in the context of both diffuse (Lifshitz et al., 2003) and focal (Singh et al., 2006a; Sullivan et al., 1999; Xiong et al., 1997a) TBI models. Moreover, the association of mitochondrial damage with LP-mediated oxidative damage is also generally known (Singh et al., 2006a; Sullivan et al., 1999; Xiong et al., 1997a), as well as the mitochondrial and neuronal protective effects of agents with direct (Deng-Bryant et al., 2008; Mustafa et al., 2010a; Xiong et al., 1997c) or indirect (Mbye et al., 2009; Mbye et al., 2008; Sullivan et al., 2011; Sullivan et al., 2000; Sullivan et al., 1999) antioxidant properties. However, the temporal pattern (i.e. onset, peak and duration) of LP-mediated oxidative damage has not been well defined in relation to the time course of mitochondrial dysfunction and neuronal degeneration. This model is a focal cortical contusion paradigm that replicates posttraumatic brain contusions that are frequently seen in moderate and severely injured TBI patients. However, in addition to the cortical contusion, its neuropathology expands over the ensuing 48–72 hours post-injury in terms of evolving neuronal and axonal degeneration both laterally and medially within the cortex and ventrally into the underlying hippocampal formation and thalamus (Hall et al., 2008). Thus, the aim of this study was to investigate the hypothesis that LP-mediated oxidative damage, mitochondrial dysfunction and neuronal cytoskeletal degradation in the brain occur in a temporally progressive, synchronous and pathophysiologically-related manner resulting in neuronal degeneration following a severe controlled cortical impact TBI (CCI-TBI) in young male rats.

Materials and Methods

Animals

Young adult male Sprague Dawley rats (total N = 127; Harlan, Indianapolis, IN; 9–10 weeks old, 300–325 g) were used for these studies. Animals had access to food and water *ad libitum*. All animal protocols met the guidelines of the NIH and complied with the policies and regulations approved by the University of Kentucky Institutional Animal Care and Use Committee (IACUC), in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals.

Materials

Sodium pyruvate, malate, rotenone, carbonyl cyanide *p*-trifluoromethoxy-phenylhydrazone (FCCP), magnesium chloride (MgCl₂), sucrose, mannitol, EGTA, EDTA, bovine serum albumin (BSA), glycerol, HEPES potassium salt, Triton-X, Tris Buffered Saline with Tween 20 (TBS-T), Tris HCl, NaCl, KCl, potassium phosphate monobasic anhydrous (KH₂PO₄), were obtained from Sigma Aldrich (St. Louis, MO); oligomycin was obtained from Enzo Life Sciences (Farmingdale, NY) and protease inhibitors (complete Mini Protease Inhibitor Cocktail tablet) were from Roche Diagnostics (Indianapolis, IN).

Rat Model of Focal (Lateral Controlled Cortical Impact) Traumatic Brain Injury (CCI-TBI)

The animals were subjected to a unilateral cortical contusion using an electronically controlled pneumatic impact device (Precision Systems & Instrumentation, Fairfax Station, VA) as previously described. All animals were anesthetized with isoflurane (2%) and placed

in a stereotaxic frame (Kopf Instruments, Tujunga, CA) the skin was retracted and a 6 mm unilateral craniotomy was performed that was centered approximately 4.5 mm between bregma and lambda. The skull cap was removed without disruption of the underlying dura. The exposed brain was injured using a 5 mm beveled tip that impacted the cortex at 3.5 m/sec to a depth of 2.2 mm. Following the injury, surgical foam (Surgicel, Johnson & Johnson) was laid upon the dura and the craniotomy site was sealed up with a sterile 8 mm plastic disc glued to the skull with Mascot tissue adhesive (Gesswein, Bridgeport, CT). The wound was closed with wound clips, the animal removed from the stereotactic device and placed in a recovery cage warmed on a circulating water heating pad until its righting reflex and full consciousness were regained before being returned to its home cage.

Isolation of Ficoll-Purified Rat Brain Cortical Mitochondria

Rat brain mitochondria were isolated from the ipsilateral (injured) cortex as previously described (Lai and Clark, 1979; Singh et al, 2013; Singh et al, 2006) at 3, 6, 12, 16, 24, 48, 72 h or 5d after injury (8–10 rats/time point) or from Sham (craniotomized, but not injured) rats at the corresponding post-surgery time points 1 or 2/time point. In brief, rats were deeply anesthetized with CO₂, and decapitated. The brains were quickly dissected to expose the cortical tissue the easily visualized injury site was collected using an 8 mm punch centered over the 5 mm diameter injury epicenter and surrounding peri-contusional cortical tissue. Brain tissue removed from the cortical punch was then homogenized using a Potter-Elvehjem homogenizer containing ice-cold isolation buffer (215 mM mannitol, 75 mM sucrose, 0.1% BSA, 20 mM HEPES, 1 mM EGTA). The homogenate was centrifuged twice at 1300 rcf for 3 min, then pelleted at 13,000 rcf for 10 min at 4°C. The crude mitochondrial pellet was re-suspended in isolation buffer and subjected to nitrogen bombing at 1200 psi and layered on top of a discontinuous 7.5% and 10% Ficoll gradient and centrifuged at 32,000 rpm for 30 min at 4°C. The final mitochondrial pellet was rinsed with isolation buffer without EGTA and then re-suspended in an appropriate volume of isolation buffer without EGTA to yield a protein concentration of 10 µg/µl. Mitochondrial protein concentration was determined using a Pierce BCA protein assay kit (ThermoFisherScientific, Omaha, NE) measuring absorbance at 562 nm with a BioTek Synergy HT plate reader (Winooski, VT). It should be noted that the final mitochondrial preparation used for bioenergetics and immunoblotting studies, described below represents total brain mitochondria which includes a mixture from neuronal, glial and vascular cell origins.

Ex Vivo Measurement of Cortical Mitochondrial Respiratory Function

Mitochondrial respiratory rates were measured using a Clarke-type electrode in a continuously stirred, sealed and thermostatically controlled chamber (Oxytherm System, Hansatech Instruments Ltd., Norfolk, England, UK) maintained at 37°C as previously described (Mustafa et al., 2010b; Singh et al., 2006b; Singh et al., 2007; Sullivan et al., 2003; Sullivan et al., 2004). Mitochondria loaded into the chamber were normalized based on protein content: Forty-100 µg aliquots of isolated mitochondrial protein were placed into the chamber with 250 µl KCl-based respiration buffer (125 mM KCl, 2 mM MgCl₂, 2.5 mM KH₂PO₄, 20 mM HEPES and 0.1% BSA, pH 7.2) warmed to 37°C and allowed to equilibrate for approximately 1 min. State II respiration was fueled by addition of the Complex I substrates, 5 mM pyruvate and 2.5 mM malate. One minute later, two boluses of

150 μ M ADP were sequentially added to the mitochondria to initiate state III respiration, followed by the addition of 2 μ M oligomycin, an inhibitor of ATP synthase, to monitor state IV respiratory rate for an additional 2 min. To assess the uncoupled respiratory rate (State V (I)), 2 μ M FCCP was added to the mitochondria, followed by the addition of 1 μ M rotenone to shut down Complex I-driven respiration completely. Complex II-driven respiration (State V (II)) was then initiated with the addition of 10 mM succinate, a Complex II substrate (Singh et al., 2006b). The slope of the oxygen consumption trace corresponded with the respiratory rate and the respiratory control ratio (RCR) was calculated by dividing the State III oxygen consumption rate (defined as the maximal rate of respiration in the presence of ADP, second bolus addition) by State IV oxygen consumption rate (defined as the rate of respiration in the presence of oligomycin, using the last 30 sec of the 2 min after the addition of the substrate). Mitochondria were isolated and prepared fresh for every experiment and were used immediately for respiration assays and then stored at -80°C for later analysis of LP-related oxidative damage.

Western Blot Measurement of Cellular Oxidative Damage in Cortical and Hippocampal Homogenates

Separate groups of sham and TBI rats were used to assess the time course of cellular oxidative damage in homogenates from the ipsilateral cortex and underlying hippocampus at 8, 16, 24 h and at 2, 3, 5 and 7 d after TBI. Similar to the methods used for the mitochondrial western blot analysis, the animals were deeply anesthetized with CO_2 , decapitated; cortical and hippocampal tissue were rapidly dissected, and immediately transferred to tubes on ice pre-filled with Triton lysis buffer with protease inhibitors. Samples were briefly sonicated, repeatedly vortexed and incubated on ice for 45 min. The samples were centrifuged at maximum speed for 30 min at 4°C and supernatants were collected for protein assay and stored at -20°C . Fifty microgram aliquots of each sample, combined with an XT Reducing Agent (Bio-Rad, Hercules, CA), were resolved on 4–12% Bis-Tris gels and transferred as described above. The membranes were incubated with the following antibodies: rabbit anti-4-HNE (ADI; 1:2,000), rabbit anti-3-NT (Upstate/EMD Millipore, Billerica, MA; 1:1,000) or rabbit anti-acrolein (Abcam; Cambridge, MA; 1:2,000) in combination with a second primary antibody, mouse anti- α -tubulin (Abcam, Cambridge, MA; 1:10,000) in TBS-T with 5% milk. After washing, the membranes were incubated with infrared labeled secondary antibodies goat anti-rabbit IRDye 800 and goat anti-mouse IRDye 680 (Li-Cor) to bind to the primary antibodies. The images were obtained using the Li-Cor Odyssey Infra-Red Imager and analyzed using Image Studio (as described above). The images were analyzed using the Odyssey Application Software (Image Studio) to obtain the integrated intensities. Alpha- or β -Tubulin western blots were done on each sample for normalization of sample loading.

Western Blot Measurement of Oxidative Damage (4-HNE) in Cortical Mitochondria

A portion of the cortical mitochondria isolated from the peri-contusional area at 3, 6, 12, 16, 24 h and 2, 3 and 5 d after TBI or from sham, non-injured rats were used for the quantitative measurements of 4-HNE as an index of LP-mediated oxidative damage. Mitochondrial samples were centrifuged at max speed for 10 min to get rid of isolation buffer. The pellets were re-suspended in 20–30 μ l ice-cold Triton lysis buffer (1% Triton-X, 20 mM tris-HCl,

150 mM NaCl, 5 mM EGTA, 10 mM EDTA, 10% glycerol) with protease inhibitors. Samples were then briefly sonicated, repeatedly vortexed, and incubated on ice for 45 minutes, then centrifuged at maximum speed for 30 min at 4°C. Supernatants were collected for protein assay and stored at -20°C.

Fifty microgram aliquots of each isolated mitochondrial sample, corresponding to the aliquots used for measurement of respiratory function, were combined with XT Reducing Agent (BioRad; Hercules, CA), resolved on 12% Bis-Tris Criterion™ XT pre-cast sodium dodecyl sulfate polyacrylamide (SDS-PAGE) gels (BioRad, Hercules, CA; 20 µl total volume) and transferred onto a nitrocellulose membrane using a semi-dry electro-transferring unit set at 15V for 60 min at 4°C. Following gel transfer, membranes were incubated in a TBS blocking solution with 5% milk for 1 hr at room temperature (RT). For detection of 4-HNE-protein conjugates, the membranes were incubated with a rabbit anti-4-HNE antibody (Alpha Diagnostics Intl. (ADI), San Antonio, TX) at a dilution of 1:2,000 in TBS-T with 5% milk on an orbital shaker overnight at 4°C. After washing with TBS and TBS-T, the membranes were incubated for an hour at RT with an infrared labeled secondary antibody goat anti-rabbit IRDye 800 (Li-Cor Biosciences, Lincoln, NE) to bind to the primary antibody. The bound complex was detected using the Li-Cor Odyssey Infrared Imaging System (Li-Cor Biosciences, Lincoln, NE). The images were analyzed using the Odyssey Application Software (Image Studio) to obtain the integrated intensities.

As noted in the section above, describing the western blot quantification of 4-HNE in brain tissue homogenates, we used α - or β -tubulin as a loading control since their quantity in injured brain homogenates because their levels were not significantly affected by TBI. However, for the 4-HNE western blots of mitochondrial proteins, we did not use β -tubulin. Although β -tubulin is known to be associated with the outer mitochondrial membrane (Kuznetsov et al., 2013) we found its content in to be too variable in our mitochondrial preparations from the injured animals to serve as a loading control. Similarly, we attempted to use the voltage-dependent anion channel (VDAC) which is the major component of the mitochondrial permeability transition pore (mPTP) and cytochrome c oxidase subunit IV (COX IV) as a loading control for mitochondrial western blotting. However, both VDAC and COX IV were also found to be effected by TBI, and thus too variable to serve as loading controls. Thus, we did not use a loading control for the mitochondrial 4-HNE quantitation. Therefore, in an effort to normalize mitochondrial protein loading across samples, we took extra care in making sure that the protein loading was equalized across gel lanes. Moreover, after transfer, the gels were stained with Coomassie blue to enable a visual estimate of equal protein loading across lanes. Lastly, since the immunoblotting studies involved multiple gels, we included a loading control in each consisting of a known concentration of 4-HNE-modified mitochondrial protein to enable quantitation across multiple blots.

Western Blot Measurement Neuronal Cytoskeletal Degradation in Cortical and Hippocampal Homogenates

For evaluation of the 150 and 145 kD α -spectrin breakdown products (SPDPs), 10 µg aliquots of each sample were used from cortical and hippocampal samples harvested from sham-non-injured rats as well as the injured hemisphere at 8, 16, and 24 h and at 2, 3, 5 and

7 d after TBI. For evaluation of the 120 kD SBDP, 50 µg aliquots (boiled for 5 minutes) of each sample were used. Prepared samples were combined with an XT Reducing Agent (BioRad), resolved on 3–8% Tris-Acetate gels and transferred as described above. The membranes were incubated with a mouse anti- α -spectrin antibody (ENZO Biochem; New York; 1:5,000) in combination with a rabbit β -tubulin (Abcam; Cambridge, MA; 1:10,000) in TBS-T with 5% milk. After washing, the membranes were incubated with infrared labeled secondary antibodies goat anti-rabbit IRDye 800 and goat anti-mouse IRDye 680 (Li-Cor Biosciences, Lincoln, NE) to bind to the primary antibodies. The images were again obtained using the Li-Cor Odyssey Infrared Imager, and analyzed using Image Studio (as described above).

Statistical Analysis

Statistical analysis was performed with Prism version 6.0 (Graph Pad, San Diego, CA). The data were analyzed using an initial one-way analysis of variance (ANOVA) followed by Student-Newman-Keuls (SNK) or Tukey post-hoc tests. For all data sets, a p-value of < 0.05 was defined as statistically significant. All data for graphical representation are expressed as mean $\pm$ standard deviation (SD).

Results

Time Course of Cortical Mitochondrial Respiratory Dysfunction After CCI-TBI

Mitochondrial bioenergetics were assessed in mitochondria isolated from the cortex of animals after a severe TBI at either 3, 6, 12, 16 and 2, 3 and 5d following injury. These analyses revealed significant differences across all states of respiration. Figure 1 demonstrates the temporal changes in mitochondrial bioenergetics following a severe CCI-TBI. Representative respiratory traces from mitochondria isolated from sham and TBI animals at 24 h and 3 days post injury (days) are shown in Figure 1A. Note the temporal deterioration in the responses to the substrates in the injured cortical mitochondria compared to sham. More specifically, Complex I of the electron transport system (ETS) is fueled by the addition of pyruvate and malate to initiate State II (Figure 1B) which significantly reduced by 3 hrs post TBI and continued to remain depressed significantly up to 5 days [ANOVA; $F = 10.1$; $df = 7, 62$; $p < 0.0001$]. The addition of ADP activates ATP synthase which initiates State III respiration. Quantification of maximal State III rates (Figure 1C) revealed a significant deficit at 3 h followed by a slight recovery out to 16 h, decreasing again at 24 h to bottom out at 3 d and trending towards recovery by 5 days [ANOVA; $F = 21.9$; $df = 7, 63$; $p < 0.0001$]. This trend was similarly seen with most of the respiratory states. State IV is initiated with the addition of oligomycin, an ATP synthase inhibitor, which shows significantly reduced responses at all time points (Figure 1D), and peaking at 3 d after TBI [ANOVA; $F = 16.9$; $df = 7, 62$; $p < 0.0001$]. FCCP is an uncoupler of the proton gradient across the inner mitochondrial membrane and the ETS then has to work maximally to make up for the gradient loss, resulting in Complex I-driven State V. Quantification of maximal State V (Complex I) oxygen consumption (Figure 1E) was significantly reduced at 3 and 12 h, while returning to sham levels at 16 h, then dropping again to maximal deficit by 3 days [ANOVA; $F = 20.8$; $df = 7, 62$; $p < 0.0001$]. The addition of rotenone, a Complex I inhibitor, followed by the addition of succinate, allows for Complex II-driven State V.

Maximal State V (Complex II) rates (Figure 1F) were significantly reduced at 3 days [ANOVA; $F = 15.3$; $df = 7, 62$; $p < 0.0001$]. The respiratory control ratio (RCR) is an index of how well the ETS is coupled to oxidative phosphorylation. Analysis of calculated RCRs (Figure 1G) shows a significant reduction at 3 days [ANOVA; $F = 11$; $df = 7, 62$; $p < 0.0001$].

In summary, the initial bioenergetic inhibitory phase at 3 h involves a 38 to 43% reduction in complex I-driven mitochondrial oxygen utilization compared to a lesser 23% attenuation of complex II-driven respiration. This is followed by a partial recovery of complex I respiratory parameters and full recovery of complex II function by 12 to 16 hours; however, thereafter there is a progressive decline in both complex I (–82%) and II (–65%)-driven oxygen utilization and a reduction in the respiratory control ratio ($RCR = -44\%$) that peaked at 3 days post-injury.

Time Course of Mitochondrial LP-Mediated Oxidative Damage After TBI

Based upon the time course of mitochondrial dysfunction, LP-mediated oxidative damage, such as 4-HNE as an index of lipid peroxidation (LP), was analyzed at 3, 6, 12, 16 and 24 h and 2, 3 and 5d post-injury time-points for ipsilateral cortical mitochondria. The accumulation of 4-HNE in isolated cortical mitochondria after CCI-TBI is demonstrated in lanes from two representative blots (Figure 2; marker lanes correspond to each gel). Western blot quantification revealed that mitochondrial 4-HNE levels progressively increased until reaching maximal levels at 3 days [ANOVA; $F = 4.89$; $df = 8, 69$; $p < 0.001$]. Due to the limited amount of mitochondrial protein, the levels of acrolein were not evaluated.

In summary, the secondary decline in mitochondrial bioenergetics between 16 hours and 3 days was paralleled by an increase in mitochondrial lipid peroxidative damage evidenced by a progressive increase in mitochondrial protein-bound 4-hydroxynonenal (4-HNE) levels which also peaked at 3 days.

Time Course of Cellular LP-Mediated Oxidative Damage After TBI

Cortical and hippocampal tissue homogenates from an additional set of sham and injured animals sacrificed at 8, 16 and 24 h, and 2, 3, 5 and 7 d after CCI-TBI were used for quantitative measurements of lipid peroxidation: 4-HNE and acrolein, as well as protein nitration (3-NT). The accumulation of 4-HNE (Figure 3A) and acrolein (Figure 3B) and 3-NT (Figure 4) are demonstrated in the representative blots (only cortical sample blots shown). As early as 8 h post injury, 4-HNE, 3-NT and acrolein levels in the injured brain were elevated and progressively increased until reaching maximal levels at 2 and 3 d following injury compared to sham levels [ANOVA; 4-HNE cortex: $F = 3.67$; $df = 7, 40$; $p = 0.004$; 4-HNE hippocampus: $F = 2.79$; $df = 7, 40$; $p = 0.018$; acrolein cortex: $F = 4.58$; $df = 7, 40$; $p = 0.001$; acrolein hippocampus: $F = 4.53$; $df = 7, 40$; $p = 0.001$; 3-NT cortex: $F = 2.88$; $df = 7, 40$; $p = 0.016$; 3-NT hippocampus: $F = 3.91$; $df = 7, 40$; $p = 0.003$]. Thus, cortical and hippocampal cellular 4-HNE and acrolein-protein binding as well as increased 3-NT levels, the latter indicative of a post-injury role of the reactive nitrogen species peroxynitrite, were also seen.

Time Course of Neuronal Cytoskeletal (α -Spectrin) Degradation After TBI

Intracellular Ca^{2+} overload resulting from PN-mediated oxidative damage and mitochondrial dysfunction activates the protease calpain. Calpain activation results in proteolytic cleavage of the neuronal cytoskeletal protein α -spectrin forming two fragments (145 kD and 150 kD) from the full length (280 kD). The 145 kD α -spectrin breakdown product (SBDP) is generated specifically by calpain, the SBDP 150 is generated by both calpain and caspase 3-mediated proteolysis, while the SBDP 120 kD is generated by caspase 3 alone. Cortical and hippocampal tissue homogenates were assessed for these SBDPs at 8, 16 and 24h, and 2, 3, 5 and 7 days after CCI-TBI as shown in the representative blot (Figure 5A). The progressive elevation of SBDP 150 in cortical samples peaks at 2 to 3 days and returns back to sham levels within the first week following TBI (Figure 5B). The trend is similar for the hippocampal samples, except that the SBDP 150 levels were elevated more quickly and remain significantly elevated out to 5d before dropping back to sham levels by 7d (Figure 5C). SBDP 145 is also progressively and significantly elevated following injury in both cortical and hippocampal samples (Figures 5D and 5E). Calpain-specific α -spectrin degradation in the injured cortex was significantly elevated at 2–3d and drops back to within normal/sham levels within the first week post TBI. In hippocampal samples, SBDP 145 progressively increased reaching peak levels at 2 days [ANOVA; 150 kD cortex: $F = 6.29$; $df = 7, 48$; $p < 0.0001$; 150 kD hippocampus: $F = 7.59$; $df = 7, 48$; $p < 0.0001$; 145 kD cortex: $F = 4.62$; $df = 7, 48$; $p = 0.0005$; 145 kD hippocampus: $F = 10.2$; $df = 7, 48$; $p < 0.0001$].

Caspase 3-specific SBDP 120 was slightly elevated at 24h; however, this was insignificant (Figure 6). Surprisingly, by 7d, the accumulation of SBDP 120 within the injured cortex had significantly dropped below sham levels [ANOVA; 120 kD cortex: $F = 5.54$; $df = 7, 40$; $p = 0.0002$]. Due to the limited change observed in cortical SBDP 120, hippocampal samples were not evaluated.

In summary, coincident with the above-described time courses of mitochondrial respiratory failure and lipid peroxidative mitochondrial and cellular oxidative damage, there was a progressive increase in calpain-mediated degradation of the neuronal cytoskeletal protein α -spectrin indicative of neuronal damage.

Discussion

Triphasic Posttraumatic Alterations in Brain Mitochondrial Bioenergetics

The results of this study describe a triphasic time course of bioenergetic functional alterations observed in cortical mitochondria removed from the injured brain at various times during the first 5 days after a severe level of CCI-TBI in male rats. As shown in Figure 1, an initial phase of mitochondrial respiratory dysfunction was observed for each of the complex I and II functional parameters measured in mitochondria removed from the injured brain at 3h after CCI-TBI. Then, by 16h after injury, there was a partial recovery of complex I-driven states II, III and IV although the oxygen utilization rates for those parameters still remained significantly below Sham levels. On the other hand, a complete recovery of complex I-driven state V and complex II-driven state V was observed by 16h. However, beginning at 24h, each of the mitochondrial respiratory parameters demonstrated a

progressive decline, all coincidentally reaching a nadir at 72h (3d) after TBI. By 72h, each oxygen consumption rate had declined more than 60% with the complex I-driven states III and V decreasing by 79% and 81%, respectively. Interestingly, the RCR, which is calculated by dividing the state III by the state 4 oxygen consumption rates, showed no posttraumatic change compared to the sham, non-injured mitochondrial RCR until 72h when it was also decreased by 44% compared to sham. This significant drop in the RCR indicates that by 72h, but not earlier, nearly half of the mitochondria in the sample were significantly uncoupled.

Interestingly, at 5d post-TBI, there was a partial recovery of the RCR as well as for each of the individual mitochondrial oxygen consumption rates (states II, III, IV, V (complex I), and V (complex II)). However, this apparent recovery does not mean that the earlier injured mitochondria have recovered their respiratory function. Rather, by 5d, the damaged and dysfunctional mitochondria are for the most part no longer present in the injured brain. Indeed, at 5d after CCI-TBI, the contusion site is fully developed (i.e. the contused cortical tissue is no longer present) (Hall et al., 2008). Thus, the 5d sampled tissue, and its mitochondria, are from surviving neural cells in the rim of tissue in periphery of the contusion site which histologically is only a hole in the cortex. Consistent with this fact is the observation that in the 5d cortical tissue sample, there is no longer a significant TBI-induced difference in oxidative damage (4-HNE) in either the isolated cortical cellular homogenate or its isolated mitochondria compared to the tissue removed from sham, non-injured mitochondria.

Role of Oxidative Damage in the Delayed Progressive Mitochondrial Failure

The time course of the secondary decline between 24 and 72h in the respiratory function of cortical mitochondria isolated from the CCI-TBI rat brains correlates well with the time course of 4-HNE accumulation in mitochondrial proteins (Figure 2) which also increases progressively between 24 and 72h. Furthermore, 4-HNE and acrolein (Figure 3) and protein nitration (Figure 4) accumulation in cortical and hippocampal cellular proteins all show that the progressive increase in oxidative damage in the injured brain parallels the decline in mitochondrial function suggesting that the increase in oxidative damage is closely linked to the secondary decline in mitochondrial respiratory function.

Prior work in our laboratory has convincingly demonstrated that the progressive post-TBI decline in mitochondrial respiratory function occurs together with an accumulation of the LP damage product 4-HNE, and the PN-mediated protein nitration product 3-NT, in mitochondrial proteins. The association of this increased oxidative damage to mitochondrial proteins being largely responsible for the post-TBI mitochondrial functional impairment is supported by the repeated finding by our laboratory or others that various antioxidant compounds have the ability to protect mitochondrial function in the acutely injured brain together with a decrease in mitochondrial and/or cellular oxidative damage. For instance, tempol, a catalytic scavenger of PN derived free radicals (Carroll et al., 2000), has been shown to protect brain mitochondrial function together with a decrease in mitochondrial protein nitration following CCI-TBI in mice (Deng-Bryant et al., 2008). Secondly, we have observed that the potent 2-methylaminochroman LP inhibitor U-83836E (Hall et al., 1991)

effectively attenuates the post-TBI accumulation of 4-HNE bound to mitochondrial proteins together with a maintenance of mitochondrial respiratory function (Mustafa et al., 2010a). Similarly, the potent pyrrolopyrimidine LP inhibitor U-101033E (Hall et al., 1997) has been demonstrated to attenuate post-TBI brain mitochondrial dysfunction (Xiong et al., 1997c). Thirdly, we have documented that the mPT inhibitor cyclosporine A (Sullivan et al., 1999) also protects mitochondrial respiratory function along with a decrease in mitochondrial protein-bound 4-HNE and 3-NT (Mbye et al., 2008) in association with an increased in spared cortical tissue and locomotor behavioral recovery (Mbye et al., 2009). Most recently, we have demonstrated that the 4-HNE scavenging agent phenelzine is also able to decrease post-TBI mitochondrial failure and 4-HNE accumulation in mitochondrial proteins along with an improvement in cortical tissue sparing in the same rat TBI model as presently employed (Singh et al., 2013). In the same study, we also showed that when non-injured Ficoll-isolated brain mitochondria are exposed *in vitro* to 4-HNE for 5 minutes this causes an impairment of respiratory parameters along with an increase in protein bound 4-HNE, whereas both are preventable by pre-exposure to the 4-HNE scavenger 4-HNE phenelzine (Singh et al., 2013).

Explanations for Early Partially Reversible Postraumatic Bioenergetic Compromise

In contrast to the association of free radical-induced oxidative damage to the secondary 24–72h decrease in mitochondrial respiratory function that reaches a maximum at 72h with a decrease in the RCR signaling that roughly half the mitochondria are uncoupled, the initial 3h posttraumatic decrease in mitochondrial respiration that recovers at least partially by 16h. For state V complex I (e.g. FCCP rate) and for state V complex II (e.g. succinate rate), these respiratory parameters recover to a degree that they are no longer different from those measured in mitochondria isolated from sham, non-injured rats. This partial inhibition of respiration is not associated with an increase in mitochondrial lipid peroxidation-derived 4-HNE modification of mitochondrial proteins. Similar results have been very recently published showing a transient decrease in mitochondrial state III respiration in mitochondria isolated at 4h post-injury from the injured cortex in rats subjected to lateral fluid percussion TBI. Similar to our results, the state III respiration rate returned to normal by 24h after injury (Ucal et al., 2017).

Multiple explanations may be singly, or collectively responsible for the early post-injury decrease in mitochondrial respiration seen at 3h after TBI. As a first possibility, in the just published lateral fluid percussion TBI study, the investigators show that their 4h partial and transient suppression of state III respiration and oxygen uptake in response to either glutamate or pyruvate state II activation was paralleled by an increase in NO[●] in the injured cortex measured by spin-trapping and electron paramagnetic resonance (EPR) (Ucal et al., 2017). This in turn appeared to be connected to a 4h increase in inducible nitric oxide synthase mRNA expression that similarly lost significance by 24h post-TBI. Additionally, the increase in NO[●] was blamed for a persistent increase in presumably PN- mediated 3-NT staining adjacent to the cortical injury site. However, no quantitation was provided (only single photomicrographs) to clearly show the magnitude of the 3-NT immunostaining across their studied time course (4h to 72 h) (Ucal et al., 2017). Thus, the magnitude of 3-NT immunostaining adjacent to their cortical injury site at 4 h compared to later timepoints

cannot be determined from their results. In contrast to their claim that the nitritative stress begins as early as 4 h, we did not see any significant increase in cortical or hippocampal 3-NT until 3 days after CCI-TBI (Figure 4), and it is likely that the 3-NT staining shown in the lateral fluid percussion TBI study is much less at 4h than it would be at later timepoints (Ucal et al., 2017). Nevertheless, our statistical analysis of the full time course is hampered by the need to correct for multiple comparisons of 7 post-TBI time points between 8h and 7 days post-injury. Indeed, visual inspection of our Figure 4 graph gives the distinct impression that the gradual increase in 3-NT begins as early as 8h after injury.

A second possible contributor to the early and transient suppression of mitochondrial respiration during the first hours following TBI seen by us and others could be due to the extensively documented massive initial influx of Ca^{2+} into brain cells secondary to initial glutamate-release and opening of Ca^{2+} gating NMDA receptor as well as Ca^{2+} entry through voltage-dependent channels (Osteen et al., 2004; Osteen et al., 2001; Sun et al., 2008; Weber, 2004). Evidence of the substantial early post-TBI accumulation of cytosolic Ca^{2+} is apparent in our time course analysis of Ca^{2+} -activated, calpain and caspase-3 mediated degradation of the cytoskeletal protein α -spectrin (Figure 5) which is statistically significant beginning as early as 8h post-injury (Figure 5C). Similarly, in the lateral fluid percussion TBI model, immunohistochemical evidence of Ca^{2+} -activated caspase 3 activation has been documented as early as 4h post-injury (Ucal et al., 2017). Therefore, the early transient partial suppression of mitochondrial respiration seen in our results, as well as in the recently published lateral fluid percussion study (Ucal et al., 2017), suggests that the early accumulation of cytosolic Ca^{2+} could be a contributor to that observation, and that free radical-induced oxidative damage does not play a significant role in mitochondrial failure until between 16 and 24h.

Consistent with this, it has recently been observed by Sullivan and coworkers (Pandya et al., 2013). that when non-injured rat brain mitochondria are exposed to increased Ca^{2+} , this produces a time- and dose-dependent inhibition of mitochondrial respiration without any significant increase in mitochondrial free radical production, protein or lipid oxidative damage or impairment of the mitochondrial respiratory enzymes pyruvate dehydrogenase or complex I-associated NADH dehydrogenase. Thus, the initial recovery of mitochondrial respiration between 3 and 16h could conceivably be occurring as mitochondrial Ca^{2+} buffering and other cellular Ca^{2+} homeostatic mechanisms (e.g. plasma and endoplasmic reticular membrane Ca^{2+} -ATPase) recover from the overwhelming injury-induced cytoplasmic Ca^{2+} influx. In contrast, the secondary decline in mitochondrial function that occurs after 16h and peaks at 72h appears to be due to gradually increasing free radical generation and accumulation of oxidative damage products. This, in turn, leads to an overwhelming exacerbation of cellular Ca^{2+} overload as mitochondrial Ca^{2+} buffering by the failing mitochondria is diminished, in addition to the compromise of other intracellular Ca^{2+} homeostatic mechanisms. This leads to a progressively increasing activation of calpain and caspase 3 that degrades neuronal cytoskeletal proteins (e.g. 280kD α -spectrin $\rightarrow$ 145 and 150 kD SBDPs) that peaks at 2–3 d after CCI-TBI (Figure 5).

Admittedly, the above scenario is inconsistent with more physiological studies that have repeatedly documented that cellular influx of Ca^{2+} within the context of normal physiology

acts to stimulate mitochondrial respiration, and that this is physiologically important for mitochondrial functional regulation and ATP production (Glancy and Balaban, 2012; Griffiths and Rutter, 2009; Llorente-Folch et al., 2013; McCormack et al., 1990). However, immediately following moderate to severe TBI, the acute influx of Ca^{2+} is supraphysiological, occurs diffusely throughout the cytosol and persists for as much as 14 days post-injury (Osteen et al., 2001). In earlier work, we have shown that mouse brain mitochondrial Ca^{2+} buffering capacity after CCI-TBI is transiently decreased by 50% as early as 3h (Singh et al., 2006a). Therefore, the compromise of Ca^{2+} homeostasis seen following moderate or severe TBI cannot be interpreted within the context our understanding of physiological intracellular Ca^{2+} regulation.

Involvement of Apoptotic Mechanisms in Delayed Phase of Progressive Mitochondrial Failure?

Interestingly, the lack of an increase in the specifically caspase 3-generated 120 kD SBDP (Zhang et al., 2009) over the first 3d (Figure 6) suggests that in the context of our presently employed severe CCI-TBI there is little contribution of apoptotically-associated caspase 3 to the progressive cytoskeletal degradation assessed in this study compared to and apparently dominant role of calpain. In fact, at 5d post-TBI, the levels of the 120 kD SBDP decreased significantly below the levels seen in Sham, non-injured, rats for which at this time, we do not have an explanation. On the other hand, the generation of the 150 kD SBDP has been shown to be partially generated by calpain and partially by caspase 3 (Zhang et al., 2009), and in our injured hippocampal showed a significant increase in the levels the 150 kD SBDP (Figure 5C) as early as 8h post-injury. Thus, caspase-3 involvement in posttraumatic cytoskeletal degradation cannot be ruled out. Furthermore, consistent with a role of apoptosis in posttraumatic mitochondrial failure, other investigators, using immortalized HT-22 hippocampal neurons, have shown that glutamate-mediated acceleration of ROS levels occurs in two phases (Tobaben et al., 2011). The first phase, which occurs at 6–8h after glutamate exposure, involves lipoxygenase (LOX) activation and its related ROS formation, but no neuronal cell death while the secondary phase which peaks at 18h of glutamate exposure, involves the activation of Bid which amplifies glutamate-mediated formation of lipid peroxides that cause irreversible mitochondrial damage involving enhanced free radical formation and apoptosis-inducing factor (AIF)-dependent cell death. Those results, obtained in hippocampal cell cultures, remarkably approximates the currently described time course of post-TBI mitochondrial failure and ROS-induced oxidative damage to mitochondria and other neural elements.

Relationship of Mitochondrial Failure Time Course in the Rat CCI-TBI Model to Human TBI

Finally, it is interesting to note that our documented time course of mitochondrial failure over the first 3 days after CCI-TBI, a model which mimics post-TBI contusions, appears to parallel the progressive mitochondrial failure seen in severe TBI patients with brain contusions at the UCLA Brain Injury Research Center. Those investigators first demonstrated, using microdialysis probes placed in the peri-contusional tissue, that the glutamate levels and the lactate/pyruvate ratio, the latter reflecting mitochondrial dysfunction were increased and that these increases were unrelated to decreases in cerebral perfusion pressure, but were associated with a reliance on anaerobic hyper-glycolysis (Vespa

et al., 2007). Subsequent efforts by the UCLA group have used [F-18] fluorodeoxyglucose and triple-oxygen positron emission tomography (PET) studies to define changes in metabolic changes in the pericontusional “penumbra” during the first 4 days after severe TBI (Wu et al., 2013). In that study, it was demonstrated that there was a progressive decline in the oxygen extraction fraction (OEF), cerebral blood flow (CBF), the cerebral metabolic rate for oxygen (CMRO₂) and the cerebral metabolic rate for glucose (CMR_{glc}) each of which were related to time after injury. This pattern of metabolic failure is consistent with progressive mitochondrial failure in the TBI patient pericontusional tissue. Furthermore, the time courses of that failure in brain tissue mitochondrial function in rats and in humans appear to be similar.

Conclusions

The current study with the widely employed rat model of CCI-TBI has documented the temporal relationship of post-TBI mitochondrial respiratory dysfunction, mitochondrial and cellular protein modification by the largely PN-derived, free radical-mediated LP-generated accumulation of 4-HNE or acrolein compared to mainly calpain-mediated neuronal cytoskeletal degradation. The results show that the time courses of the measured secondary injury parameters are similar, and that each does not peak until 48 to 72h after CCI-TBI. These findings of a surprisingly delayed peak in secondary injury, suggest that the therapeutic window and needed treatment duration for certain antioxidant treatment strategies following CCI-TBI in rodents may be longer than previously believed. Indeed, This is supported by our past therapeutic window studies in the mouse CCI-TBI paradigm in which we used attenuation of calpain-mediated α -spectrin degradation as a neuroprotective endpoint with which we observed significant cytoskeletal protective efficacy with the LP inhibitor U-83836E (Mustafa et al., 2011), the cyclosporine A analog NIM811 (Mbye et al., 2009) and the Nrf2-antioxidant response element activator carnosic acid (Miller et al., 2015) even when dosing was not initiated until as much as 12h after TBI. Additionally, in the rat CCI-TBI paradigm we observed a significant increase in cortical spared tissue at one week post-injury could be achieved with cyclosporine A treatment even when treatment initiation was delayed for as long as 8h (Sullivan et al., 2011). Moreover, the doses of each drug that protected against cytoskeletal breakdown had been shown earlier to optimally reduce post-TBI mitochondrial respiratory dysfunction and lipid peroxidative accumulation of 4-HNE which further supports the connection between PN-induced LP damage, mitochondrial dysfunction and cytoskeletal proteolytic degradation. The similarity of the rat and human mitochondrial failure time courses may be an indicator that the therapeutic window for pharmacologically protecting aerobic brain function after TBI might be achievable in humans as well as in rodents.

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Highlights

1. A biphasic time course of brain mitochondrial failure is provided over the first 5 days after controlled cortical impact TBI in adult male rats.
2. The initial phase at 3 hour involves a 43% reduction in complex I oxygen utilization which partially recovers by 12 h.
3. Beginning at 16h there is a progressive decline in complex I oxygen utilization that peaks at 3 days post-injury.
4. Coincident with the above-described time course, there was a progressive increase in calpain-mediated degradation of the neuronal cytoskeletal protein α -spectrin.
5. The temporal linkage of these post-TBI events suggests a possible neuroprotective therapeutic window of as much as 12 to 24 hours.

Figure 1.

Time course of cortical mitochondrial respiratory dysfunction during the first 5 days after CCI-TBI: Following an initial decrease in oxygen utilization at 3h post-TBI, complex I and II respiration partially recovers after which there is a progressive secondary decline that peaks at 3 days. The apparent recovery at 5 days is due to the fact that most of the damaged mitochondria have deteriorated to the point that only the uninjured mitochondria are included in the Ficoll isolation as shown previously (Singh et al., 2006a). Representative traces from the Oxytherm (A) illustrate the progressive decline in respiratory capacity over

time in the rates of oxygen consumption between mitochondria isolated from injured cortices compared to Sham (non-injured) cortex. Oxygen consumption rates for each state of mitochondrial respiration were quantified and representative graphs (B – G) demonstrate the temporal profile of mitochondrial dysfunction following TBI (n = 8 – 10/injury group/time point; sham (n = 10); * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001; SNK, compared to Sham).

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Figure 2.

Time course of cortical mitochondrial LP-related accumulation of 4-HNE modified proteins during the first 5 days after CCI-TBI: Increased level of 4-HNE peaks at 3 days parallels the peak of respiratory dysfunction shown in Fig. 2. Similarly, the decline in 4-HNE at 5 days post-TBI mimics the seeming recovery in respiratory dysfunction in Fig. 2. The western blot shows the differences over time in the accumulation of 4-HNE in mitochondrial proteins. Quantification of 4-HNE bands between 150 – 50 kD reveals significant, progressive temporal elevation in 4-HNE accumulation is significant as 2 and 3 days. Primary antibody: rabbit anti-4-HNE (1:2000, ADI), normalized to sham levels (n = 10/group, except for 3day group (n = 8); ** p < 0.01, *** p < 0.001, **** p < 0.0001; SNK, compared to Sham).

Figure 3.

Time course of LP-related accumulation of 4-HNE and acrolein-modified proteins in cortex and hippocampus during the first 5 days after CCI-TBI. **A) 4-HNE:** The western blot shows the difference over time in the accumulation of cellular 4-HNE within the injured cortex. Quantification of 4-HNE bands between 150 – 50 kD reveals progressive temporal elevation in 4-HNE accumulation in the injured cortex and hippocampus. Primary antibodies: rabbit anti-4-HNE (1:2000, ADI) with reducing agent, normalized to mouse β -tubulin (1:10,000, AbCam; n = 6/group; * $p < 0.05$, compared to sham (cortex) and 16h group (hippocampus); SNK). **B) Acrolein:** Representative blot shows temporal differences in accumulation of acrolein within the injured cortex and hippocampus. Quantification (100-50 kD) reveals progressive temporal accumulation of acrolein in both cortical and hippocampal tissue peaking at 2 and/or 3 days post-injury. Primary antibodies: rabbit anti-acrolein (1:2000, AbCam) with reducing agent, normalized to mouse α -tubulin (1:10,000, AbCam; n = 6/group). Cortical samples: ** $p < 0.01$, *** $p < 0.001$, compared to sham; hippocampal samples: *** $p < 0.001$, compared to sham SNK.

Figure 4.

A) The western blot shows a representative blot demonstrating the differences over time in protein nitration within the injured cortex. Quantification of 3-NT immunoreactivity between 150 – 50 kD once again shows progressive accumulation of 3-NT in cortex and hippocampus that peaks at 3 days. Primary antibodies: rabbit anti-3-NT (1:1000, Upstate) with reducing agent, normalized to mouse α -tubulin (1:10,000, AbCam; n = 6/group). Cortical samples: ** p < 0.01, compared to sham; hippocampal samples: *** p < 0.001, compared to sham; (SNK).

Figure 5.

Western blot shows temporally progressive accumulation of the 150 and 145 kD α -spectrin breakdown products (note the bands at the arrows) within the injured cortex. Quantification of 150 kD and 145 kD bands in cortical and hippocampal tissue samples revealed significant increases in SBDP accumulation over time. In the injured cortex, SBDP peaked at 2–3 days, while in the underlying hippocampus (C, E), SBDP levels were already significantly elevated by 8h and remained elevated until 5 days. Primary antibodies: mouse anti- α -spectrin (1:5000, ENZO) with reducing agent, normalized to rabbit β -tubulin (1:10,000, AbCam; $n = 10$ /group for sham and 3d groups; all others 6/group. All analyses were by one way ANOVA followed by SNK post-hoc testing comparing injured to sham: Cortical samples: 150 kD bands: **** $p < 0.0001$, * $p < 0.05$, compared to sham; 145 kD bands: *** $p < 0.001$, * $p < 0.05$, compared to sham; Hippocampal samples: 150 kD bands: ** $p < 0.01$, **** $p < 0.0001$; 145 kD bands: ** $p < 0.01$, **** $p < 0.0001$.

0.05, compared to sham; Hippocampal samples: 150 kD bands: **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, compared to sham; 145 kD bands: **** $p < 0.0001$, ** $p < 0.01$, compared to sham.

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Figure 6.

A) Representative blot shows no significant accumulation of the 120 kD SBDP (note the band at the arrow) within the injured cortex; however, there was a significant reduction by 7 days. Primary antibodies: mouse anti- α -spectrin (1:5000, ENZO) with reducing agent, normalized to rabbit β -tubulin (1:10,000, AbCam; n = 6/group); ** p < 0.01, compared to sham.