Novel Differential Neuroproteomics Analysis of Traumatic Brain Injury in Rats*

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Approximately two million traumatic brain injury (TBI) incidents occur annually in the United States, yet there are no specific therapeutic treatments. The absence of brain injury diagnostic endpoints was identified as a significant roadblock to TBI therapeutic development. To this end, our laboratory has studied mechanisms of cellular injury for biomarker discovery and possible therapeutic strategies. In this study, pooled naïve and injured cortical samples (48 h postinjury; rat controlled cortical impact model) were processed and analyzed using a differential neuroproteomics platform. Protein separation was performed using combined cation/anion exchange chromatography-PAGE. Differential proteins were then trypsinized and analyzed with reversed-phase LC-MSMS for protein identification and quantitative confirmation. The results included 59 differential protein components of which 21 decreased and 38 increased in abundance after TBI. Proteins with decreased abundance included collapsin response mediator protein 2 (CRMP-2), glyceraldehyde-3-phosphate dehydrogenase, microtubule-associated proteins MAP2A/2B, and hexokinase. Conversely, C-reactive protein, transferrin, and breakdown products of CRMP-2, synaptotagmin, and αII-spectrin were found to be elevated after TBI. Differential changes in the above mentioned proteins were confirmed by quantitative immunoblotting. Results from this work provide insight into mechanisms of traumatic brain injury and yield putative biochemical markers to potentially facilitate patient management by monitoring the severity, progression, and treatment of injury. Molecular & Cellular Proteomics 5:1887–1898, 2006.

Traumatic brain injury (TBI), defined as brain damage due to mechanical force applied to the head, has an annual economic cost of $65 billion in the United States (1). There are over two million TBI incidents with ~500,000 hospitalizations and 100,000 deaths annually (2–5). TBI is particularly prevalent among the young; it is considered the leading cause of death and disability among children and young adults. Despite these facts, there are no specific therapeutic treatments for TBI.

TBI is difficult to assess by current clinical techniques such as magnetic resonance imaging and computer tomography. Surrogate markers such as brain temperature, oxygen level, and pressure lack sensitivity, specificity, and availability (5–7). There is thus a need for a sensitive and specific biochemical marker(s) of TBI with the diagnostic ability to evaluate postconcussive intracranial pathology to improve patient management and facilitate therapeutic evaluation (6). In particular altered neurodegenerative or protective proteins could be of great value if they could provide insight into injury severity and outcome (8). A small number of TBI protein markers have been reported including lactate dehydrogenase, glial fibrillary acid protein, enolase, and S-100B; however, all lack either the necessary sensitivity, TBI specificity, or both to be exclusively effective (5, 7, 9, 10). Furthermore the biochemical mechanism that produces post-TBI changes in these proteins are not understood, leaving them as potential surrogate markers rather than true biochemical markers of known injury pathways. To this end, breakdown products of proteolyzed proteins are of particular interest in neurotrauma as they provide a direct assessment of a known neurodegenerative mechanisms with the potential for therapeutic intervention.

Following TBI, there is a shift in the balance between pro- and anti-apoptotic protein machinery promoting either cell survival or death (11–13). Studies reported from our and other laboratories provided substantial evidence for the involvement of overactivated cysteine proteases as major intracellular effectors of neuronal cell death via both necrotic and apoptotic pathways (5, 14). The primary mechanical injury produces a robust pattern of necrotic cell death in close proximity to the impact site that is mediated by calpains, protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CRMP-2, collapsin response mediator protein 2; MAP, microtubule-associated protein; BDP, breakdown product; 1D, one-dimensional; 2D, two-dimensional.
calcium-activated cysteine proteases implicated in oncosis (14). Czogalla and Sikorski (14) in a recent review stressed, with high emphasis on trauma-related pathology, this pivotal role of calpain in neurodegenerative disease. However, secondary insults often involve apoptotic cell death in regions caudal to the impact site. Apoptosis involves complex cascading pathways resulting in the activation of executioner proteases such as caspase-3 by intrinsic and extrinsic mechanisms involving caspases-8 and -9 (11, 15). Caspase-3 then acts on a number of cytosolic and cytoskeletal neuronal substrates, for example the cytoskeletal protein \( \alpha \)-II-spectrin, which upon proteolysis yield signature breakdown products (BDPs) that are indicative of neuronal cell death dynamics (4–6, 14, 16–18).

Recently proteomics has been identified as a potential means for biomarker discovery with the ability to identify proteome dynamics in response to experimental stimuli (3, 8, 19–23). Gel electrophoresis with or without cyanine dye labeling is often used for protein separation and differential selection prior to mass spectrometry (8, 16, 17, 24). Shortcomings of gel-based approaches can include limited resolution, mass range, and reproducibility (3, 25). For example, in a previous TBI study, we utilized 1D DIGE protein separation in series with reversed-phase liquid chromatography tandem mass spectrometry peptide analysis as a means to discover putative TBI biomarkers (3). However, the limited protein separation confounded the results. Subsequently we developed a novel multidimensional protein separation and differential analysis platform, comprised of the steps depicted in Fig. 1, to improve differential protein identification and overcome some of the limitations observed in 1D and 2D DIGE (25). Importantly, the platform involves correlating semiquantitative peptide data with gel densitometry data to reduce false positives during differential analysis. Our hypothesis is that the cation/anion exchange (CAX) chromatography-PAGE/reversed-phase (RP) LC-MSMS (CAX-PAGE/RPLC-MSMS) platform will improve discovery of differential protein changes post-TBI and facilitate discovery of biochemical markers and possible therapeutic interventions.
Experimental Procedures

Brain Tissue Collection and Protein Extraction—All procedures involving animal handling and processing were done in compliance with guidelines set forth by the University of Florida Institutional Animal Care and Use Committee and the National Institutes of Health guidelines. A controlled cortical impact device was used to model TBI in male Sprague-Dawley rats as described elsewhere (26). TBI was performed on seven animals, each mounted in a stereotactic frame and impacted in the right cortex (ipsilateral) with a 5-mm-diameter aluminum impactor tip at a velocity of 3.5 m/s to a depth of 1.6 mm. Seven naive control animals were kept under the same environmental conditions but did not receive an impact injury. At 48 h postinjury, naive and injured animals were sacrificed by decapitation. TBI and naive cortex tissues were homogenized using a small mortar and pestle set over dry ice. The homogenized cortical tissue powder was then lysed for 90 min at 4 °C with a 0.1% SDS lysis buffer containing 150 mM sodium chloride, 1% ethoxylated octylphenol, 1 mM sodium vanadate, 3 mM EDTA, 2 mM EGTA, 1 mM dithiothreitol (all from Sigma) with a Complete Mini protease inhibitor mixture tablet containing EDTA along with a mixture of a broad spectrum of serine, cysteine, metalloprotease, and calpain inhibitors suited for animal tissues (Roche Applied Science). Brain cortex lysates were then centrifuged at 16,000 g for 10 min at 4 °C. The supernatant was retained and collected at 4 °C to prevent proteolysis. The protein content was determined using the DC protein assay (Bio-Rad) after which the protein concentration was standardized to 1 μg/μl for immunoblotting analysis.

Combined CAX-PAGE—The CAX chromatography was performed on a Bio-Rad Biologic DuoFlow system with sulfopropyl- (S1) and quaternary ammonium-(Q1) modified Sepharose prepacked ion-exchange columns (Bio-Rad) connected in tandem along with a Quad-Tec UV detector and BioFrac fraction collector. A detailed description of the CAX chromatography setup was described recently (25). For the purpose of this study, proteins from the sacrificed rats (n = 7) were pooled to amass the required amount of protein and average inconsistent protein levels due to biological variability. Protein concentration of the seven TBI cortical samples was determined, and 0.143 mg of protein from each tissue sample was pooled to constitute 1 mg of protein, which was loaded on the liquid chromatography system. A pooled naive sample (1 mg of protein, n = 7) was similarly produced. A total of 32 1-ml fractions were collected during CAX chromatography, each concentrated using Millipore YM-10 ultrafiltration units (Millipore Corp., Bedford, MA) according to the manufacturer’s instructions. Laemmli sample buffer (25 μl) was then added to the YM-10 collection filters and incubated for 10 min prior to collection by centrifugation at 1000 × g for 3 min. Protein fractions were run side-by-side (i.e., naive fraction 1 next to TBI fraction 1, etc.) using 18-well, 10–20% gradient Tris-HCl Bio-Rad Criterion gels for differential comparison of TBI and naive samples. ImageJ software was used for quantitative densitometric analysis of select gel band intensities. Differential bands were boxed and labeled according to their 2D position (e.g., the top band excised from the lane of fraction 6 was labeled 6A).

Western Blot Analysis and Antibodies—Four naive and four TBI samples were processed with 2× Laemmli sample buffer (Bio-Rad with 5% β-mercaptoethanol). 20 μg of protein from each sample was subjected to gel electrophoresis on 10–20% or 6% Tris-glycine gels and then transferred onto PVDF membranes. Following the transfer, the membranes were blocked in 5% nonfat dry milk for an hour and then incubated overnight with the primary antibody at 4 °C. On the following day, the membranes were washed three times with 1× Tris-buffered saline/Tween 20 and probed with the secondary antibody for an hour. Immunoreactivity was detected by using a streptavidin-alkaline phosphatase-conjugated tertiary antibody. Monoclonal anti-mouse αII-spectrin (Affiniti Research Products, Ltd.) and anti-β-actin (Sigma) were used at a dilution of 1:4000 in 5% milk. Antibodies for profilin (BD Transduction Laboratories), hexokinase (Chemicon International, Temecula, CA), anti-MAP2A/2B (BD Pharmingen), anti-synaptotagmin (Abcam Ltd., Cambridge, UK), anti-GAPDH (EnCor Biotechnology, Alachua, FL), anti-collin (Cell Signaling Technology, Beverly, MA), anti-C-reactive protein (R&D Systems, Minneapolis, MN), anti-chicken polyclonal transferrin (Abcam Ltd.), and anti-col-

![CAX chromatography overlay of TBI and naive chromatograms. CAX chromatographic separations of naive and TBI pooled rat cortical lysates (n = 7) are shown overlaid with the same 280 nm absorbance scale: naive in black and TBI in gray. Thirty-two fractions were collected from each separate run.](Image)
lapsin response mediator protein 2 (CRMP-2) (Immuno-Biological Laboratories Co., Ltd.) were used at a dilution of 1:1000 in 5% milk. Secondary biotinylated antibody (Amersham Biosciences) and streptavidin-alkaline phosphatase-conjugated tertiary antibody (Amersham Biosciences) were used at a dilution of 1:3000 in 5% milk.

**Gel Band Visualization and Quantification—** ImageJ densitometry software (Version 1.6, National Institutes of Health, Bethesda, MD) was used for gel band quantitative densitometric analysis. Selected bands were quantified based on their relative intensities. -Fold increase or decrease between naïve and TBI samples was calculated by dividing the greater value by the lesser value with a negative sign to indicate a decrease after TBI.

**Statistical Analysis of Western Blotting Data—** Densitometric quantification of the immunoblot bands was performed using an Epson Expression 8836XL high resolution flatbed scanner (Epson, Long Beach, CA) and ImageJ densitometry software. Densitometry values of four replicates of naïve and TBI samples were evaluated for statistical significance with SigmaStat software (Version 2.03, Systat Software Inc.) and a Student's t test. A p value of <0.05 was considered to be significant for data acquired in arbitrary density units.

**In-gel Digestion and Reversed-phase Liquid Chromatography Tandem Mass Spectrometry—** A detailed description of the RPLC-MSMS platform has been described elsewhere (25). In brief, differential bands were excised, cut into pieces, and washed with HPLC water (Burdick & Jackson, Muskegon, MI) followed by 50:50 100 mM ammonium bicarbonate:acetonitrile (Burdick & Jackson, HPLC grade). Bands were dehydrated with 100% acetonitrile, then rehydrated with 10 mM DTT for 30 min at 56 °C, and then alkylated with 55 mM iodoacetamide in 50 mM ammonium bicarbonate for 30 min in the dark at room temperature followed by acetonitrile dehydration. For protein digestion, 15 μl of a 12.5 ng/μl trypsin solution was added and incubated for 30 min at 4 °C. An additional 20 μl of 50 mM ammonium bicarbonate was then added, and that mixture was incubated overnight at 37 °C. The resulting peptide solution was collected and hydrophobic peptide extraction performed with 50:50 water:acetonitrile. The peptide solution was dried by speed vacuum, and the residue was suspended in mobile phase solution for RPLC-MSMS analysis. Capillary reversed-phase liquid chromatography tandem mass spectrometry protein identification was performed by loading 2 μl of sample digest via autosampler onto a 100-μm × 5-cm C_18 reversed-phase capillary column at 1.5 μl/min. Peptides were eluted via a linear gradient: 5–60% methanol in 0.4% acetic acid over 30 min at 500 nl/min. Tandem mass spectra were collected using a data-dependent method (three most intense peaks) on a Thermo Electron LCQ Deca XP Plus ion trap mass spectrometer (Thermo Electron, San Jose, CA). Protein database searching of tandem mass spectra was performed against a National Center for Biotechnology Information (NCBI) rat-indexed RefSeq protein database using Bioworks Browser (Version 3.1, Thermo Electron). Subtractive filtering and sorting were performed with DTAselct software (Version 1.9, The Scripps Research Institute) on singly, doubly, and triply charged tryptic peptides with a cross-correlation (Xcorr) value greater than 1.8, 2.5, and 3.5.

**Fig. 3.** Comparison of rat naïve and TBI CAX fractions via side-by-side SDS-PAGE separation. One milligram of cortical pooled rat TBI and naïve lysates was sequentially separated by CAX-PAGE into 32 fractions. Shown is the side-by-side (naïve on the left; TBI on the right) pairing of 29 of the 32 fractions run on 1D PAGE. Selected bands are boxed and labeled with letters and numbers according to their gel position, i.e. a band with a 6A label represents the first top band excised from lane 6. These bands are then excised for subsequent RPLC-MSMS identification. N, naïve; I, injury.
respectively. Naïve and TBI data were then compared with the Contrast module of the DTAselect software (27).

**Semiquantitative Differential Correlation of Protein and Peptide Data**—The number of identified peptides per protein was tabulated from the filtered Bioworks data for naïve and TBI gel band pairs. Naïve and TBI peptide numbers were compared; those identified proteins with a two or more difference in the number of peptides were retained. The greater peptide number must then correlate with the sample (naïve or TBI) demonstrating the greater gel band density to be considered a differential protein (Tables I and II). We previously reported a correlation rate of 89% utilizing these parameters (25).

**RESULTS**

**CAX-PAGE Neuroproteomics Experimental Design**—This study utilized a novel neuroproteomics approach as outlined in the systemic seven-step process illustrated in Fig. 1 comprising the multidimensional neuroproteomics platform. Our experimental design called for two pooled rat samples: injured ipsilateral cortical lysate from 48-h post-TBI animals and control naïve ipsilateral cortical lysate from uninjured animals. The protein components from each sample were differentially resolved by a two-dimensional protein separation technique termed CAX-PAGE. Naïve and TBI lysates were sequentially separated by CAX chromatography based on protein charge; the two chromatograms are shown overlaid in Fig. 2. The initial impression after the first dimension separation was that there is a marked difference between the two proteomes, reminiscent of the difference between cortex and cerebellum tissues observed in our first report on CAX-PAGE separation (25). In this first study, the coefficient of variation value for repeated CAX chromatography separation was determined to be 11% with no discernable variation in the chromatographic trace. Thus the disparate chromatograms in Fig. 2 are attributed to the alteration of the cortical proteome associated with the TBI insult.

Thirty-two fractions collected from each CAX chromatography experiment were paired (i.e., each CAX fraction 1 from control was paired with fraction 1 of TBI) and loaded side-by-side onto a 1D polyacrylamide gel for the second dimension protein separation. The gels were visualized with Coomassie Blue stain for differential band analysis. Thirty-one bands with an observed difference in densitometry were selected and excised for differential band analysis. Thirty-one bands with an observed difference in densitometry were selected and excised for differential band analysis. Thirty-one bands with an observed difference in densitometry were selected and excised for differential band analysis. Thirty-one bands with an observed difference in densitometry were selected and excised for differential band analysis. Thirty-one bands with an observed difference in densitometry were selected and excised for differential band analysis. Thirty-one bands with an observed difference in densitometry were selected and excised for differential band analysis.

**Identification of Differential Proteins by RPLC-MSMS**—Following CAX-PAGE separation, the differential bands were processed for peptide separation and analysis by RPLC-MSMS. Tandem mass spectra were searched using Bioworks Browser against a rat-indexed protein database revealing between zero and four proteins per gel band, each having two or more peptides. For those bands with multiple identified proteins we utilized the number of matched peptides per protein as a semiquantitative measure of protein abundance to confirm which protein represents the observed differential gel pattern as illustrated by Peng et al. (28). Using the peptide data we were able in most cases to isolate a single protein.

**Table I**

| Band | Gel M, kDa | Intact molecular mass | Protein accession no. | Protein name | Naïve No. pep. | TBI No. pep. | Cov. |
|------|-----------|-----------------------|----------------------|---------------|---------------|--------------|-----|
| 6A   | 56,000    | 72.1                  | XP_237959            | Annexin A11   | 6             | 0            | 11.0 |
| 6B   | 20,000    | 18.5                  | AAH86533             | Aldehyde dehydrogenase family 7 | 3 | 1 | 7.4 |
| 8A   | 15,000    | 14.9                  | NP_071956            | Coflin 1      | 5             | 3            | 28.3 |
| 9B   | 56,000    | 57.8                  | AAB93667             | M2 pyruvate kinase | 15 | 12 | 29.8 |
| 9C   | 55,000    | 50.9                  | XP_227366            | α-Enolase (non-neural enolase) | 2 | 0 | 7.05 |
| 9D   | 50,000    | 47.1                  | AAH78896             | M2 pyruvate kinase | 7 | 2 | 15.40 |
| 9E   | 34,000    | 35.8                  | XP_573896            | Glyceraldehyde-3-phosphate dehydrogenase | 5 | 1 | 23.0 |
| 10A  | 105,000   | 102.4                 | NP_036866            | Hexokinase 1 | 4 | 0 | 5.5 |
| 10B  | 85,000    | 85.4                  | NP_077374            | Aconitase 2, mitochondrial | 7 | 1 | 11.2 |
| 10C  | 72,000    | 74.8                  | XP_215897            | Acetyl-CoA synthetase 2 | 3 | 0 | 10.4 |
| 10D  | 21,000    | 22.4                  | AAL66341             | Neuronal protein 22 | 3 | 0 | 18.6 |
| 12A  | 45,000    | 44.8                  | AAH83568             | Phosphoglycerate kinase 2 | 4 | 0 | 10.8 |
| 13A  | 70,000    | 70.4                  | CAA49670             | Phosphoglycerate kinase 1 | 5 | 0 | 13.2 |
| 13B  | 58,000    | 61.3                  | NP_036702            | Hsc70-ps1 | 11 | 5 | 22.6 |
| 13C  | 37,000    | 39.3                  | NP_036627            | Glutamate dehydrogenase 1 | 4 | 0 | 8.6 |
| 13D  | 34,000    | 31.1                  | NP_036629            | Aldolase A | 3 | 0 | 9.3 |
| 17A  | 64,000    | 62.2                  | NP_071633            | Aldolase C, fructose-biphosphatase | 4 | 0 | 16.5 |
| 23A  | 200,000   | 182.2                 | NP_037198            | Dimethylarginine dimethylaminohydrolase 1 | 3 | 1 | 10.5 |
|      |           |                       |                     | Collapsin response mediator protein 2 | 7 | 4 | 15.9 |
|      |           |                       |                     | Microtubule-associated protein 2 | 5 | 1 | 3.4 |
that matched the gel data. In a few cases, two or more proteins produced differential peptide numbers as reported in Tables I and II. In all, 59 proteins were confirmed by this process to have a different abundance between naïve and TBI samples. The identified proteins were grouped as having decreased (21 proteins) or increased (38 proteins) abundance post-TBI. The proteins that decreased post-TBI included the cytosolic glycolytic proteins glycerdehyde-3-phosphate dehydrogenase, enolase, aldehyde dehydrogenase, glutamate dehydrogenase, and hexokinase; the cytoskeleton-associated proteins profilin and cofilin; and the neuron-specific proteins CRMP-2 and neuronal protein 22 (Table I) (29, 30). Among the TBI increased proteins are the glycolytic proteins lactate dehydrogenase, brain creatine kinase, and malate dehydrogenase; the ubiquitin-associated proteins UCH-L1 and proteasome subunit α type 7; the cytosolic cell signaling protein 14-3-3 family members; and the serum-derived proteins transferrin, C-RP, ferroxidase, albumin, fetuin, hemoglobin, and serine protease inhibitors (Table II).

**Validation of Proteins with Decreased Abundance after TBI—** Five of the proteins decreased in abundance after TBI were subjected to biochemical validation by Western blotting: cofilin, profilin, GAPDH, hexokinase, and MAP2A/2B protein (Fig. 5). Protein selection was based on several factors including antibody availability, literature relevance, and levels of peptide abundance. Based on these criteria several other proteins remain to be validated. Validation by this means is presently the bottleneck in biomarker development where the discovery rate exceeds the rate of preliminary validation by severalfold (31). Densitometric analysis showed a statistically

| Band | Gel Mr. | Protein accession no. | Protein name | No. pep. | Cov. % | Naïve | TBI | Cov. |
|------|---------|-----------------------|--------------|----------|--------|-------|-----|------|
| 1A   | 31,000  | XP_226922             | Carbonic anhydrase | 3        | 6      | 30.0  |
| 6B   | 20,000  | NP_543180             | ADP-ribosylation factor 3 | 1        | 3      | 17.7  |
| 7A   | 75,000  | NP_058751             | Transferrin | 0        | 8      | 13.2  |
| 8A   | 15,000  | XP_340780             | Hemoglobin α chain | 0        | 5      | 33.8  |
| 9A   | 77,000  | AAP79736              | Liver regeneration-related protein | 0        | 2      | 15.0  |
| 9B   | 56,000  | NP_445800             | Fetuin β | 0        | 4      | 11.6  |
| 9E   | 34,000  | XP_227088             | 3-Oxoadic-CoA transferase | 1        | 4      | 10.4  |
| 36.4 | NP_150238 | Malate dehydrogenase 1, NAD (soluble) | 0        | 2      | 5.7    |
| 36.6 | NP_036727 | Lactate dehydrogenase B | 1        | 4      | 13.8   |
| 35.6 | AAP63165 | Malate dehydrogenase, mitochondrial | 0        | 2      | 7.6    |
| 72,000 | 75.8     | NP_058751 | Transferrin | 0        | 3      | 13.2  |
| 60.1 | JX0054 | Carboxylesterase E1 precursor | 0        | 5      | 13.6   |
| 59.9 | XP_227088 | 3-Oxoadic-CoA transferase | 1        | 4      | 10.4   |
| 41.5 | NP_445800 | Fetuin β | 0        | 4      | 11.8   |
| 20,000 | 20.6     | NP_058751 | ADP-ribosylation factor 3 | 1        | 3      | 17.7  |
| 15.2 | XP_340780 | Hemoglobin α chain | 0        | 5      | 33.8   |
| 15.9 | NP_150237 | Hemoglobin β chain | 0        | 2      | 15.0   |
| 76.7 | AAP79736 | Liver regeneration-related protein | 0        | 2      | 2.6    |
| 55.9 | XP_227088 | 3-Oxoadic-CoA transferase | 1        | 4      | 10.4   |
| 36.4 | NP_150238 | Malate dehydrogenase 1, NAD (soluble) | 0        | 2      | 5.7    |
| 36.6 | NP_036727 | Lactate dehydrogenase B | 1        | 4      | 13.8   |
| 35.6 | AAP63165 | Malate dehydrogenase, mitochondrial | 0        | 2      | 7.6    |
| 72,000 | 75.8     | NP_058751 | Transferrin | 0        | 3      | 13.2  |
| 60.1 | JX0054 | Carboxylesterase E1 precursor | 0        | 5      | 13.6   |
| 59.9 | XP_227088 | 3-Oxoadic-CoA transferase | 1        | 4      | 10.4   |
| 41.5 | NP_445800 | Fetuin β | 0        | 4      | 11.8   |
| 20,000 | 20.6     | NP_058751 | ADP-ribosylation factor 3 | 1        | 3      | 17.7  |
| 15.2 | XP_340780 | Hemoglobin α chain | 0        | 5      | 33.8   |
| 15.9 | NP_150237 | Hemoglobin β chain | 0        | 2      | 15.0   |
| 76.7 | AAP79736 | Liver regeneration-related protein | 0        | 2      | 2.6    |
| 55.9 | XP_227088 | 3-Oxoadic-CoA transferase | 1        | 4      | 10.4   |
| 36.4 | NP_150238 | Malate dehydrogenase 1, NAD (soluble) | 0        | 2      | 5.7    |
| 36.6 | NP_036727 | Lactate dehydrogenase B | 1        | 4      | 13.8   |
| 35.6 | AAP63165 | Malate dehydrogenase, mitochondrial | 0        | 2      | 7.6    |

**Table I**

**Proteins with increased abundance post-TBI**

No. pep., number of peptides; Cov., sequence coverage. *(BDP) denotes a suspected breakdown product.*
FIG. 4. Differential gel band analysis. Using ImageJ densitometry software, differential gel band intensities of naïve and TBI were quantified to derive the relative -fold increase and decrease. Quantitative densitometric analysis was performed on the selected bands based on their relative intensities. Sixteen gel bands were found to have more than a 2-fold increase; 13 gel bands were found to have more than a 2-fold decrease. N, naïve; I, injury.

FIG. 5. Western blot validation of TBI decreased proteins identified by mass spectrometry in individual naïve and TBI cortex samples (n = 4). Shown is Western blot analysis of intact 15-kDa profilin, 120-kDa hexokinase, 19-kDa cofilin, 36-kDa GAPDH, and 200-kDa MAP2A/2B proteins comparing four individual naïve samples with four individual TBI samples. These blots show lower protein abundance in the four TBI samples compared with the naïve samples. Western blot data are suggestive of either down-regulation or degradation post-TBI insult. Graphical representation of the densitometric analysis using ImageJ software from the Western blot data shows decreased protein abundance (profilin, hexokinase, cofilin, GAPDH, and MAP2A/2B). Naïve samples are represented by open bars, and TBI samples are represented by dotted bars. Student’s t test was performed to evaluate statistical significance (*, p < 0.05; mean ± S.E.M.; n = 4). Data are expressed in arbitrary units. N, naïve; I, injury.
significant decrease of cofilin, profilin, hexokinase, GAPDH, MAP2A/2B, and intact CRMP-2 proteins (p < 0.05; Student’s t test) in TBI samples relative to naïve.

Validation of Proteins with Increased Abundance after TBI—Similar to the decreased protein validation, a targeted approach was applied in selecting and validating a number of the proteins that increased after TBI including C-reactive protein and transferrin (31). Densitometric analysis showed a statistically significant increase of C-RP and transferrin proteins (p < 0.05; Student’s t test) in TBI relative to naïve samples (Table II). All were characterized via Western blot, which showed the same molecular mass shift observed in the proteomics data (Fig. 7). Confidence for our data came from the co-migration of the suspected αII-spectrin breakdown product (intact mass, 280 kDa) along with the 120-kDa proteins ferroxidase and ceruloplasmin in gel band 20A that aligned with an observed molecular mass of 120 kDa. The immunoblotting data confirmed the increase in the 120-kDa spectrin breakdown product (Table II and Band 20A). Similarly CRMP-2 (intact mass, 62 kDa) co-migrated with GDP dissociation inhibitor-1 (intact mass, 51 kDa) and group-specific component protein (intact mass, 53 kDa) in gel band 18B that aligned with an observed molecular mass of 54 kDa (Table I and Band 18B). The immunoblotting data confirmed the increase in a 54-kDa CRMP-2 BDP. Densitometric data indicated that the increase in αII-spectrin, synaptotagmin, and CRMP-2 breakdown products was statistically significant after TBI (p < 0.05; Student’s t test) relative to naïve samples.

Fig. 6. Western blot validation of TBI increased proteins identified by mass spectrometry in individual naïve and TBI animals (n = 4). Shown is Western blot analysis of intact 75-kDa transferrin and intact 25-kDa C-RP comparing four individual TBI samples with four individual naïve samples. These blots show higher protein abundance in the four TBI samples compared with the four naïve samples. Western blot data are indicative of blood-brain barrier disruption along with inflammatory process occurring within injured brain tissues. Graphical representation of the densitometric analysis using ImageJ software shows elevated TBI protein (transferrin and C-RP). Student’s t test was performed to evaluate statistical significance (*, p < 0.05; mean ± S.E.M.; n = 4). Data are expressed in arbitrary units. Western blot of β-actin served as a loading control and shows equal loading in both conditions. Naïve samples are represented by open bars, and TBI samples are represented by dotted bars. Graphical representation of densitometric data shows no statistical significance difference (*, p < 0.05; mean ± S.E.M.; n = 4). N, naïve; I, injury.
DISCUSSION

The CAX-PAGE/RPLC-MSMS neuroproteomics platform, a multidimensional separation technique (Fig. 1), was applied to identify proteome changes in rat cortex 48 h post-TBI, providing more definitive results through better proteome separation and quantitative validation than from our earlier study (3). To do this, the altered TBI proteome is contrasted against a naïve cortical proteome. In total, 59 proteins showed an altered abundance post-TBI (Tables I and II) and were divided into three groups: decreased, increased, or putatively degraded by proteolysis (Figs. 5–7).

The proteins with decreased abundance post-TBI (Table I) were the result of changes in expression, cellular metabolism, and/or proteolytic degradation. Included in this group are the cytoskeleton-associated proteins, cofilin (Band 6B), profilin (Band 8A), MAP2A/2B (Band 23A), and hexokinase (a cytoplasmic phosphotransferase, Band 10A), all of which were validated to decrease after TBI by immunoblotting (Fig. 5). Also the data revealed a decrease in GAPDH after TBI (Band 9E), denoting the loss of metabolic function. Importantly GAPDH is widely regarded as an unchanging housekeeping protein used as a loading control in Western blots; however, this would be inappropriate in neurotrauma studies given the data in Fig. 5. Post-TBI GAPDH dynamics should also be

![Graphical representation of the densitometric analysis using ImageJ software of the four individual naïve and TBI Western blot data shows potential TBI breakdown products of αII-spectrin, CRMP-2, and synaptotagmin identified by mass spectrometry post-TBI. Student’s t test was performed to evaluate statistical significance (*, p < 0.05; mean ± S.E.M.; n = 4). Data are expressed in arbitrary units. Naïve samples are represented by open bars, and TBI samples are represented by dotted bars. N, naïve; I, injury.](image-url)
considered in light of its emerging role as a proapoptotic enzyme that induces nuclear translocation in a number of neurodegenerative diseases (30, 32). Among other interesting proteins is NP22, a neuronal protein that mediates interactions between cytoskeletal proteins (33). Unfortunately NP22 was one of the proteins that we could not confirm by immunoblotting due to the lack of an available antibody.

Proteins with increased abundance following TBI (Table II) were either up-regulated or accumulated in response to injury (Fig. 6) (2). The rapid and long term accumulation of proteins in reaction to axonal injury within different neuronal compartments has already been reported post-TBI and is evident in our study (2). Increased proteins included members of the acute phase protein family, which are indicative of an inflammatory response (34, 35). The observed acute phase proteins were C-reactive protein, transferrin, and ceruloplasmin, which were all validated by immunoblotting to increase in individual animals. Additional proteins validated by immunoblotting to increase 48 h after TBI included \( \alpha_1 \) inhibitors and kininogen proteins. The increased high molecular mass \( \alpha_1 \) inhibitor ceruloplasmin, not observed with alternative 2D DIGE separation, indicated blood-brain barrier leakage (36). Increased abundance of kininogen protein C-RP, a member of the thiostatin family, is indicative of inflammatory processes, shown previously to be of clinical importance following TBI and ischemic stroke (34, 37). The results correspond well with known post-TBI pathology, which involves inflammation coupled with a breakdown in the blood-brain barrier, leading to the extravasations of plasma proteins (38). Other non-inflammatory proteins that increased after TBI include (Table II) UCH-L1 (Band 13E), lactate dehydrogenase (Band 9E), and members of the \( \alpha_2 \) spectrin family (nominally 280 kDa, Band 18A) appeared as a 120-kDa spectrin breakdown product following caspase-3 proteolysis during apoptosis (5, 10). These data correlate with our previous findings of differential proteome changes providing complementary side-by-side fraction comparison allows for direct visualization of differential proteome changes providing complementary data to the pi shifts observed by 2D DIGE (25).

Of interest is the specificity of the different proteins identified, which included a number of brain-specific proteins (synaptotagmin, CRMP-2, NP22, MAP2, and brain creatine kinase). However, due to the complexity of the nervous system, the dynamic nature of the proteome expression in general, and its dependence on various signals (insults, development, etc.), these proteins can reflect signal-dependent neuroanatomical specificity. One example is the NP22 protein, which shows expression among various brain regions. However, in alcoholism, NP22 would show an increased expression in the frontal cortex but not in other brain regions (33). Similarly our current work identified a number of brain-specific proteins; however, it was not feasible to validate that these proteins are actually cortex-specific especially given that relevant literature does not specify brain-specific anatomical expression of such proteins but rather considers them to be ubiquitous in expression throughout the brain (NP22, synaptotagmin, and CRMP-2). Interestingly, in a previously published work from our group we compared protein expression between rat cerebellar and cortex regions (25). In this study, the proteomic map reflected cortex-dominant proteins (MAP2 and \( \alpha \)-enolase) and cerebellum-dominant proteins (14-3-3...
protein family), whereas brain creatine kinase was comparable in both regions. In our current study, TBI reflected the dynamic nature of protein expression rendering the expression of the 14-3-3 and brain creatine kinase to be elevated in TBI shifting the basal cortical map proteomic pattern. Thus, among the currently identified proteins, the brain-specific proteins can be considered cortex-specific under TBI insult. Nevertheless further studies are needed to evaluate the expression of these proteins in different neuroanatomical areas under the same condition.

The initial goal for differential TBI neuroproteomics analysis using the CAX-PAGE/RPLC-MSMS platform is to identify likely biomarker candidate proteins, which will subsequently be validated by immunological studies in biological fluids of animals and eventually humans. Inherent to biomarker development is the likelihood that putative protein markers may not be adequately detected in biological fluids and prove suboptimal as clinical diagnostics (19, 45). Due to the time-consuming nature of devising and optimizing enzyme-linked immunoassay for each putative biomarker protein, it is critical to reduce the number of false positives identified by proteomics as differentially altered after TBI. To this end, we performed a secondary quantitative evaluation step, utilizing peptide data to correlate protein abundance with densitometry data. Reported (Tables I and II) are those proteins having a two or more difference in the number of identifying peptides (naïve versus TBI) with the greater number in the gel band with the larger optical density. Thereby those identified proteins that reportedly do not demonstrate a measurable difference between naïve and TBI samples likely are not differential in nature, or those that do not correlate with the densitometry data are not reported. In our previous report of the differential platform, 89% of selected differential gel bands had correlating differential peptide data. The described quantitative correlation process effectively reduced the number of false-positive differential proteins reported (and subsequently developed into assays) as evident from our immunological validation work where six of seven putative markers tested were confirmed as differential in multiple naïve and TBI samples. The importance of this process cannot be overstressed as all multidimensional protein separation techniques lack the necessary resolving capability to produce fractions, spots, or bands that routinely contain only a single protein. The approach, however, may increase the number of false negatives as the acquired peptide data is semiquantitative in nature. It is also anticipated that other differential proteins are not targeted for mass spectrometry analysis as they do not produce a differential band density of 2-fold or greater, further confounded by the presence of multiple proteins in a single band; hence the reported list is not exhaustive in nature. Rather our intention for this study is to identify those differential proteins that are dramatic in nature to be developed into biochemical markers of TBI whereby an exhaustive differential study is not time-efficient. Beyond biomarker discovery, the identified differential protein changes reflect injury mechanisms that with further study may be relevant to therapeutic intervention, such as the systematic inhibition of proteolytic activity at discrete time points post-TBI. For this purpose, other differential analysis techniques, such as ICAT or 2D PAGE methods, can be used in combination with CAX-PAGE/RPLC-MSMS to provide greater coverage of the altered TBI proteome for more detail on mechanisms of cellular injury and death, barring in mind that no one technique can effectively capture an entire proteome (for more on this topic, see Ref. 16 for our recent review).

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REFERENCES

1. NINDS (2006) Traumatic Brain Injury: Hope Through Research, NIH Publication Number 02-2478, National Institutes of Health, Bethesda, MD
2. Smith, D. H., Uryu, K., Saatman, K. E., Trojanowski, J. Q., and McIntosh, T. K. (2003) Protein accumulation in traumatic brain injury. Neuromol. Med. 4, 59–72
3. Haskins, W. E., Kobeyssi, F. H., Wolper, R. A., Ottens, A. K., Kitten, J. W., McClung, S. H., O’Shee, B. E., Chow, M. M., Pineda, J. A., Denslow, N. D., Hayes, R. L., and Wang, K. K. (2005) Rapid discovery of putative protein biomarkers of traumatic brain injury by SDS-PAGE-capsillary liquid chromatography-tandem mass spectrometry. J. Neurotrauma 22, 629–644
4. Farkas, O., Polgar, B., Szekeres-Bartho, J., Doczi, T., Povlishock, J. T., and Buki, A. (2005) Spectrin breakdown products in the cerebrospinal fluid in severe head injury—preliminary observations. Acta Neurochir. (Wien) 147, 855–861
5. Pineda, J. A., Wang, K. K., and Hayes, R. L. (2004) Biomarkers of proteolytic damage following traumatic brain injury. Brain Pathol. 14, 202–209
6. Ingebrietsen, T., and Romner, B. (2003) Biochemical serum markers for
brain damage: a short review with emphasis on clinical utility in mild head injury. Restor. Neurol. Neurosci. 21, 171–176
7. Pelsers, M. M., Hermens, W. T., and Glatz, J. F. (2005) Fatty acid-binding proteins as plasma markers of tissue injury. Clin. Chim. Acta 352, 15–35
8. Denslow, N., Michl, M. E., Temple, M. D., Hsu, C. Y., Saatman, K., and Hayes, R. L. (2003) Application of proteomics technology to the field of neurotrauma. J. Neurotrauma 20, 401–407
9. Bandypopadhyay, S., Hennes, H., Gorelick, M. H., Wells, R. G., and Walsh-Kelly, C. M. (2005) Serum neuron-specific enolase as a predictor of short-term outcome in children with closed traumatic brain injury. Acad. Emerg. Med. 12, 732–738
10. Siman, R., McIntosh, T. K., Soetse, K. M., Chen, Z., Lewis, S. B., Meegan, C., Oli, M. W., Roberts, V. L., and McIntosh, T. K. (2004) Proteins released from degenerating neurons are surrogate markers for acute brain damage. Neurobiol. Dis. 16, 311–320
11. Lotocki, G., Alonso, O. F., Frydel, B., Dietrich, W. D., and Keane, R. W. (2005) The use of proteomics in biomarker discovery of rat TBI.
12. McDonald, W. H., and Yates, J. R., III (2002) DTASelect and Contrast: tools for assembling and comparing protein identifications from shotgun proteomics. J. Proteome Res. 1, 21–26
13. Peng, J., Kim, M. J., Cheng, D., Duong, D. M., Gygi, S. P., and Sheng, M. (2004) Semiquantitative proteomic analysis of rat forebrain postsynaptic density fractions by mass spectrometry. J. Biol. Chem. 279, 21002–21011
14. Liu, X., Crain, D. F., Krueckenbauer, K., and Fountoulakis, M. (2003) Proteomics in brain research: potentials and limitations. Prog. Neurobiol. 69, 193–211
15. Jenkins, L. W., Peters, G. W., Dixon, C. E., Zhang, X., Clark, R. S., Skinner, J. C., Marion, D. W., Adelson, P. D., and Kochanek, P. M. (2002) Conventional and functional proteomics using large format two-dimensional gel electrophoresis 24 hours after controlled cortical impact in postnatal day 17 rats. J. Neurotrauma 19, 715–740
16. Bodovitz, S., and Joos, T. (2004) The proteomics bottleneck: strategies for overcoming an independent validation of potential biomarkers and drug targets. Trends Biotechnol. 22, 4–7
17. Hara, M. R., Agrawal, N., Kim, S. F., Cascii, M. B., Fujimuro, M., Ozeki, Y., Takahashi, M., Cheah, J. H., Tankou, S. K., Hester, L. D., Ferris, C. D., Hayward, S. D., Snyder, S. H., and Sawa, A. (2005) S-Nitroslated GAPDH initiates apoptotic cell death by nuclear translocation following Sia1l binding. Nat. Cell Biol. 7, 665–674
18. Fan, L., Jaquet, V., Dodd, P. R., Chen, W., and Wilce, P. A. (2001) Molecular cloning and characterization of NNP22: a gene up-regulated in human alcoholic brain. J. Neurochem. 76, 1275–1281
19. Kosinska, K. (1992) Serum C-reactive protein monitoring in children after injuries of closed body cavities. Ann. Acad. Med. Stet. 38, 67–78
20. Sironi, L., Tremoli, E., Miller, I., Guerini, U., Calvo, A. M., Eberini, I., Gemeiner, M., Ascente, M., Paolietti, R., and Gianazza, E. (2001) Acute-phase proteins before cerebral ischemia in stroke-prone rats: identification by proteomics. Stroke 32, 753–760
21. Sironi, L., Guerini, U., Tremoli, E., Miller, I., Gelosa, P., Lascialfari, A., Zucca, I., Eberini, I., Gemeiner, M., Paolietti, R., and Gianazza, E. (2004) Analysis of pathological events at the onset of brain damage in stroke-prone rats: a proteomics and magnetic resonance imaging approach. J. Neurosci. Res. 78, 115–122
22. Di Napoli, M., Papa, F., and Bocca, V. (2001) C-reactive protein in ischemic stroke: an independent prognostic factor. Stroke 32, 917–924
23. Liu, H. M., and Sturner, W. Q. (1988) Extravasation of plasma proteins in stroke: an independent prognostic factor. Stroke 19, 285–295
24. Pipe, B. R., Flint, J., Dutta, S., Johnson, E., Wang, K. K., and Hayes, R. L. (2001) Accumulation of non-erythroid II-spectrin and calpain-cleaved alpha II-spectrin breakdown products in cerebrospinal fluid after traumatic brain injury in rats. J. Neurochem. 78, 1297–1306
25. Czech, T., Yang, J. W., Csaszar, E., Kappler, J., Baumgartner, C., and Lace, G. (2004) Reduction of hippocampal collagen response mediated protein-2 in patients with mesial temporal lobe epilepsy. Neurochem. Res. 29, 2189–2196
26. Casteaga, A., Aksenov, M., Thongboonkerd, V., Klein, J. B., Pierce, W. M., Booze, R., Markesbery, W. R., and Butterfield, D. A. (2002) Proteomic identification of oxidatively modified proteins in Alzheimer’s disease brain. Part II: dhypdyrominidase-related protein 2, α-enolase and heat shock cognate 71. J. Neurochem. 82, 1524–1532
27. Liu, M. C., Akle, V., Zheng, W., Dave, J. R., Tortella, F. C., Hayes, R. L., and Wang, K. K. (2006) Comparing calpain- and caspase-3-mediated degradation patterns in traumatic brain injury by differential proteome analysis. Biochem. J. 394, 715–725
28. Quilbibi, G. A., Gong, J. H., Tam, E. M., McCulloch, C. A., Clark-Lewis, I., and Overall, C. M. (2000) Inflammation dampened by gelatinase A cleavage of monocoye chemoattractant protein-3. Science 289, 1202–1206
29. Lopez-Ortin, C., and Overall, C. M. (2002) Prostate degradomics: a new challenge for proteomics. Nat. Rev. Mol. Cell Biol. 3, 509–519
30. Downey, T., Coon, K. D., and Stephan, D. A. (2005) Discovery and development of biomarkers of neurological disease. Drug Discov. Today 10, 326–334