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Research paper

Identification of differentially expressed proteins in rats with spinal cord injury during the transitional phase using an iTRAQ-based quantitative analysis

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ABSTRACT

Background: Spinal cord injury (SCI) is a disease associated with high disability and mortality rates. The transitional phase from subacute phase to intermediate phase may play a major role in the process of secondary injury. Changes in protein expression levels have been shown to play key roles in many central nervous system (CNS) diseases. Nevertheless, the roles of proteins in the transitional phase of SCI are not clear.

Methods: We examined protein expression in a rat model 2 weeks after SCI and identified differentially expressed proteins (DEPs) using isobaric tagging for relative and absolute protein quantification (iTRAQ). Gene Ontology (GO) analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of DEPs were performed. Furthermore, we constructed a protein-protein interaction (PPI) network, and the top 10 high-degree core nodes were identified. Meanwhile, we validated protein level changes of five high-degree core regulated proteins using Western blots.

Results: A total of 162 DEPs were identified between the injury group and the control, of which 101 (62.35%) were up-regulated and 61 (37.65%) were down-regulated in the transitional phase of SCI. Key molecular function, cellular components, biological process terms and pathways were identified and may be important mechanisms in the transitional phase of SCI. *Alb*, *Calm1*, *Vim*, *Apoe*, *Syp*, *P4hb*, *Cd68*, *Eef1a2*, *Rab3a* and *Lgals3* were the top 10 high-degree core nodes. Western blot analysis performed on five of these proteins showed the same trend as iTRAQ results.

Conclusion: The current study may provide novel insights into how proteins regulate the pathogenesis of the transitional phase after SCI.

Abbreviations: SCI, Spinal cord injury; CNS, central nervous system; DEPs, differentially expressed proteins; iTRAQ, isobaric tagging for relative and absolute protein quantification; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; PPI, protein-protein interaction; GFAP, glial fibrillary acidic protein; STRING, The Search Tool for the Retrieval of Interacting Genes; Alb, serum albumin; Calm1, calmodulin; Vim, vimentin; Apoe, apolipoprotein E; Syn, synaptophysin; P4hb, protein disulfide-isomerase; Cd68, the Cd68 molecule; Eef1a2, elongation factor 1-alpha 2; Rab3a, Ras-related protein Rab-3A; Lgals3, galectin; GABA, Gamma-aminobutyric acid; CSPGs, Chondroitin sulfate proteoglycans; RPTPσ, receptor protein tyrosine phosphatase sigma; CaMKII, Calcium/calmodulin dependent kinase II

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1. Introduction

Spinal cord injury (SCI) is a disease associated with high rates of disability and mortality. It is often caused by high-energy damage, especially falls and traffic accidents (Jain et al. 2015). SCI first causes neurological damage, such as neuronal necrosis, and subsequently leads to a series of secondary injuries, such as free radical formation, lipid peroxidation and the inflammatory response (Silva et al. 2014), and reactive astrogliosis, which also creates an environment that prevents axon growth (Profyris et al. 2004). These injuries may result in long-term tissue destruction and function loss. The secondary injury is usually divided into several phases: the acute phase, the subacute phase, the intermediate phase and the chronic phase. The definition of phases is controversial: some studies define the subacute phase as occurring days to weeks after SCI (Norenberg et al. 2004; Silva et al. 2014), while others suggest that the subacute phase lasts from 2 days to 2 weeks post-injury, and the intermediate phase lasts from 2 weeks to 6 months (Rowland et al. 2008; Ahuja et al. 2017a; Ahuja et al. 2017b). According to these, we defined 2 weeks post SCI as a transitional phase. In this phase, reactive astrocytes at the periphery of the injury site become hypertrophic and proliferative, and the expression of glial fibrillary acidic protein (GFAP) actively increases to form glial scars (Rowland et al. 2008), presenting both physical and chemical barriers to axonal regeneration that may limit functional recovery. The transitional phase may be a key therapeutic time point for further study. Although numerous treatment methods, such as cell therapy, have been proposed, examined and found to exert positive effects, an effective and generally accepted cure for SCI is not available (Huang et al. 2015; Assinck et al. 2017). Therefore, explorations of protein expression in the transitional phase are urgently needed to establish differentially expressed protein (DEP) profiles and obtain a better understanding of the molecular mechanisms of SCI to identify better therapeutic targets and measures.

Proteomics is the study of the composition, distribution and interaction of proteins throughout a whole cell or organism. The use of this technique to analyze and evaluate DEPs may help researchers thoroughly explore the pathophysiological mechanisms of SCI, systematically study the relationship between proteins and corresponding signaling pathways, identify new biological markers and provide more detailed evaluation criteria for protein levels to evaluate prognoses (Parker et al. 2010). Recently, isobaric tagging for relative and absolute protein quantification (iTRAQ) has been proven to be a highly sensitive quantitative proteomics approach for the identification and quantification of proteins (Wiese et al. 2007). The advantages of iTRAQ are its high throughput, high stability and lack of restrictions on sample properties, and up to 8 samples have been analyzed simultaneously using a commercially available kit (Craft et al. 2013).

In the present study, we used iTRAQ to examine protein expression levels 14 days post-SCI compared with controls. The identified DEPs may be critical in the transitional phase. Our findings may describe the proteomic profile of the transitional phase from the subacute to the intermediate phase of SCI. Moreover, our findings may be beneficial to a better understanding of the potential molecular mechanisms of and will be useful in the future exploration of effective therapeutic targets for SCI.

2. Materials and methods

2.1. Animals

Adult female Wistar rats (200–230 g) were obtained from the Academy of Military Medical Sciences (Tianjin, China). All procedures involving animals were approved by the Ethics Committee of Tianjin Medical University and were conducted in accordance with the Guidance Suggestions for the Care and Use of Laboratory Animals from the Ministry of Science and Technology of China. All rats were allowed to adapt to the preoperative environment for 1 week with normal circadian rhythms and were given free access to water and food.

2.2. Spinal cord contusion injury

Rats were randomly divided into two groups: one group did not undergo surgery prior to extraction (control group) and one group underwent a T10 contusive SCI (injured group). Each group contained 9 animals. Laminectomy was performed at the level of the 10th thoracic vertebra (T10), and contusive SCI was induced using a New York University (NYU) impactor device (10 g weight at a vertical height of 25 mm, 10 g × 25 mm). In all cases, both hindlimbs of the rat twitched involuntarily and the tail waggled after SCI, indicating that the injuries to the rats were in accordance with the criteria of the SCI model, as previously described (Ban et al. 2009). The animals' bladders were manually evacuated twice a day postoperatively. The rats in the injured group were sacrificed at 2 weeks post-SCI, and a 10-mm spinal cord segment containing the injury epicenter was removed for iTRAQ analysis. Spinal cord tissues at T10 were also removed from control rats at 2 weeks after laminectomy.

2.3. Protein extraction from spinal cord tissues

The 10-mm spinal cord samples from both experimental and control groups were preserved at −80 °C. Each sample included the spinal cords of three rats and weighed approximately 200 mg. Three hundred microliters of lysis buffer (8 M urea, 50 mM Tris, pH 8.0, 1% NP40, 1% NaDOC (Sodium deoxycholate), 2 mM EDTA (Ethylenediaminetetraacetic acid), 10 mM DTT (Dithiothreitol), and 1% proteinase inhibitor) were added to each sample, followed by homogenization at 800 rpm. Samples were subjected to ultrasonic dissolution on ice. Centrifugation was performed at 20,000*g* for 10 min at 4 °C, and the intermediate layer was transferred to a new tube (the upper layer contained a white lipid). Then, a 2D Quant kit (GE Healthcare) was used to determine the protein concentrations of the intermediate layer solutions after repeated centrifugation. Thirty micrograms of protein were extracted from each sample and separated via 12% SDS-PAGE. The same strip distribution and staining degree were used for each sample to examine the accuracy of the parallel and quantitative protein extraction results.

2.4. iTRAQ labeling

Five hundred micrograms of protein were extracted from each sample, and lysis buffer was used to supplement the difference in volume. A final concentration of 5 mM DTT (Solarbio, Biotechnology) was used to reduce disulfide bonds, and the reaction was performed at 30 °C for 40 min. A 40 mM IAM solution (Sigma, BioUltra) was added to quench free sulfhydryl groups after cooling to room temperature, and the reaction was performed in the dark at room temperature for 40 min. An 8-fold volume of acetone that had been pre-cooled at −20 °C was added to precipitate the protein, followed by incubation overnight at −20 °C. Samples were centrifuged at 20,000*g* for 10 min at 4 °C, and the supernatant was discarded; 80% acetone that had been pre-cooled at −20 °C was used for rinsing, followed by incubation for 1 h at −20 °C. Similarly, samples were rinsed twice with pre-cooled acetone, and the supernatant was removed. Proteins were precipitated for 10 min at room temperature to volatilize the remaining acetone. Then, 300 μl of 0.1 M TEAB (Sigma, BioUltra) was added, followed by ultrasonic dissolution of the precipitated protein on ice. Next, 5 μg of trypsin was added to promote enzymolysis and incubated overnight at 37 °C. Finally, 5 μg of trypsin was added, followed by enzymolysis for 4 h at 37 °C, and TFA (Sigma, HPLC) was added to a final concentration of 1% to terminate the reaction. A C18 SPE (Phenomenex, 10 mg/ml) column was used for desalting, and the eluted peptide fragments were dried with a vacuum concentrator. The peptide fragments were dissolved in