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Proteomic analysis of PSD-93 knockout mice following the induction of ischemic cerebral injury

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ABSTRACT

Postsynaptic density protein-93 (PSD-93) is enriched in the postsynaptic density and is involved in *N*-methyl-D-aspartate receptor (NMDAR) triggered neurotoxicity through PSD-93/NMDAR/nNOS signaling pathway. In the present study, we found that PSD-93 deficiency reduced infarcted volume and neurological deficits induced by transient middle cerebral artery occlusion (tMCAO) in the mice. To identify novel targets of PSD-93 related neurotoxicity, we applied isobaric tags for relative and absolute quantitative (iTRAQ) labeling and combined this labeling with on-line two-dimensional LC/MS/MS technology to elucidate the changes in protein expression in PSD-93 knockout mice following tMCAO. The proteomic data set consisted of 1892 proteins. Compared to control group, differences in expression levels in ischemic group >1.5-fold and <0.66-fold were considered as differential expression. A total of 104 unique proteins with differential abundance levels were identified, among which 17 proteins were selected for further validation. Gene ontology analysis using UniProt database revealed that these differentially expressed proteins are involved in diverse function such as synaptic transmission, neuronal neurotransmitter and ion transport, modification of organelle membrane components. Moreover, network analysis revealed that the interacting proteins were involved in the transport of synaptic vesicles, the integrity of synaptic membranes and the activation of the ionotropic glutamate receptors NMDAR1 and NMDAR2B. Finally, RT-PCR and Western blot analysis showed that SynGAP, syntaxin-1A, protein kinase C β, and voltage-dependent L-type calcium channels were inhibited by ischemia–reperfusion. Identification of these proteins provides valuable clues to elucidate the mechanisms underlying the actions of PSD-93 in ischemia–reperfusion induced neurotoxicity.

1. Introduction

Cerebral ischemia–reperfusion induced neuronal injury involves a complex signal transduction network, and its mechanisms remain incompletely understood. Such injury is initiated by the release and aggregation of excitatory amino acids during ischemic brain damage (Brassai et al., 2014). Consequently, *N*-methyl-D-aspartate receptors (NMDA receptors or NMDARs) are activated by postsynaptic density (PSD) scaffolding proteins, leading to a series of pathological changes (Zhang et al., 2007, 2010; Zhou et al., 2010; Xu et al., 2004). NMDARs are tetrameric complexes consisted of several subunits. The subunit composition of NMDARs is plastic, leading to a large number of NMDAR

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subtypes. Up to now, seven different subunits have been identified, including GluN1 subunit, four distinct GluN2 subunits (GluN2A, GluN2B, GluN2C and GluN2D), and two GluN3 subunits (GluN3A and GluN3B) (Paoletti et al., 2013). Notably, during early postnatal development, NMDARs switch subunit composition from primarily containing GluN2B subunits to predominantly containing GluN2A subunits in central nerve system, coinciding with the processes of synapse maturation and learning (Dumas, 2005).

PSD scaffolding proteins play a vital role in NMDAR activation-induced ischemic brain injury. PSD-93 is a PSD scaffolding protein that belongs to the membrane-associated guanylate kinase family (MAGUKs). PSD-93 plays key role in synaptic development, synaptic plasticity and the scaffolds of postsynaptic complexes (Jiang et al., 2003; Zheng et al., 2011). Moreover, several studies suggest that PSD-93 might be involved in chronic pain, morphine tolerance, morphine withdrawal and learning and memory dysfunction via its effects on synaptic plasticity (Liaw et al., 2008; Tao et al., 2010; Zhang et al., 2003). In addition, PSD-93 is implicated in ischemic brain injury. PSD-93 knockout mice might engage the hypoxic-ischemic injury induced NMDAR signaling pathway by upregulating the protein expression of PSD-95 (Jiang et al., 2003). Recently, our study revealed that PSD-93 interacted with nNOS and NR2A to promote nNOS activation via platelet-activating factor (PAF) to mediate ischemic brain injury (Xu et al., 2004). Additionally, PSD-93 knockout significantly increased the proliferative capacity of cells, which reduces the neuronal death that results from ischemic brain injury (Zhang et al., 2010). Furthermore, PSD-93 knockout exhibited neuroprotective effects against ischemic brain injury that are mediated by the inhibition of the Fyn-mediated phosphorylation of NR2B (Zhang et al., 2014). These findings suggest that PSD-93 plays an important role by suppressing ischemia-induced neuronal excitotoxicity during ischemic brain injury, but the exact mechanisms of this function require further exploration.

Recent advances in proteomics technology have enabled the application of a sophisticated labeling-based quantitative method based on iTRAQ labeling coupled with on-line two-dimensional LC/MS/MS technology to provide important insights into the pathogenesis of major psychiatric and neurodegenerative disorders and ischemic brain injury in patient cohorts (Martins-De-Souza et al., 2010; Johnston-Wilson et al., 2000; Pennington et al., 2008; Castegna et al., 2002) and animal models (Ditzen et al., 2006; Mu et al., 2007; Otte et al., 2011; Patel et al., 2007; Robinson et al., 2011; Szego et al., 2010). Although previous studies have shown that PSD-93 is involved in the neuronal excitotoxicity induced by ischemic brain injury, no studies have attempted to detect the proteomic changes associated with PSD-93 disruption and excitotoxicity.

In the present study, iTRAQ-based quantitative proteomics analysis was applied to identify potential protein targets of PSD-93 disruption in adult mouse transient middle cerebral artery occlusion (tMCAO) model. To further explore the mechanisms responsible for the function of PSD-93 in MCAO induced excitotoxicity, 17 target proteins were selected for gene ontology (GO) function and network analyses. We found that the ras GTPase-activating protein SynGAP (SynGAP), isoform 2 of the voltage-dependent L-type calcium channel subunit beta-4 (VDLC), syntaxin-1A, and PKCβ are involved in PSD-93 mediated neurotoxicity.

2. Materials and methods

2.1. Animals

PSD-93 knockout (KO) mice (C57BL/6 genetic background) were produced as described previously (Paoletti et al., 2013). Male PSD-93 KO mice and wild-type (WT) littermates (10–12 weeks old) were obtained by interbreeding PSD-93 heterozygous mice. The genotype of each mouse was confirmed by polymerase chain reaction. All animal experiments were approved by the Institutional Animal Care and Use Committee of Nanjing University. Mice were housed in cages individually with free food and water supply. The environment was at a temperature ($23 \pm 2\,^{\circ}C$), humidity (40–60%) and a 12/12 h dark–light cycle (light on 9:00–21:00).

2.2. MCAO model

Transient focal cerebral ischemia was induced as previously described (Zhang et al., 2012). Briefly, PSD-93 KO mice and WT C57BL/6 mice were anesthetized by intraperitoneal injection of ketamine (100 mg/ml) and xylazine (20 mg/kg) mixture (1:1) at a dose of 1 ml/kg. A 6/0 monofilament nylon suture with a heat-rounded tip was inserted through the internal carotid artery into the beginning of the right middle cerebral artery (MCA). The mice were subjected to 2 h of occlusion, and blood flow was then restored by the withdrawal of the filament at 4, 24, 48, or 72 h. Sham control animals were subjected to similar operations without the MCA occlusion. During the experiment, the body temperature was maintained at $37 \pm 0.5\,^{\circ}C$ with an infrared heating lamp. The mortality of both PSD-93 KO mice and WT C57BL/6 mice in the model group was approximately 10%. During the reperfusion, post-operative mice had adequate water and food and 24 h awakening-sleep cycle. Those paralysis mice accepted special care and artificial feeding at least 3 times a day.

2.3. Infarct size measurement

After anesthesia using sodium pentobarbital, three PSD-93 KO mice and three WT C57BL/6 mice in the model group were executed by cervical dislocation and quickly decapitated at the indicated reperfusion time, respectively. The brains were washed with freezing PBS solution, and then cut into five 2-mm-thick slices and stained with 2,3,5-triphenyltetrazolium chloride (TTC, Sigma) at $37\,^{\circ}C$ for 20 min in the dark to detect infarct volume. Images were acquired with a computer-controlled digital camera (Olympus) and analyzed with Image-ProPlus 6.0 (IPP) software (Media Cybernetics) to calculate the infarct sizes. The infarct volume in all slices was expressed as the percentages of the contralateral hemisphere after correcting for edema.

2.4. Behavioral test

Neurological Severity Score (NSS) is a composite of motor, sensory, reflex, and balance tests and was evaluated at 4, 24, 48, and 72 h after MCAO (Chen et al., 2001). Neurological function was graded on a scale ranging from 0 to 18 (normal score, 0; maximal deficit score, 18). Higher scores indicated more severe injury (normal score: 2–3; maximal deficit score: 18).

2.5. Quantitative proteomic analysis

Quantitative proteomic analysis was performed with iTRAQ labeling (Applied Biosystems) coupled with an on-line two-dimensional nanoLC/MS/MS system (2D-nanoLC-MS/MS) (Agilent, Waldbronn, Germany). Briefly, each treatment group ($n = 5$) was labeled with iTRAQ reagents (114:WT sham, 115:WT R4 h, 116:PSD-93-/- sham and 117:PSD-93-/- R4h) mixed with 100 μg of protein. The mixed samples were cleaned, desalted and vacuum-dried and subsequently analyzed with an on-line two-dimensional nano LC/MS/MS on a nano-HPLC system coupled with a hybrid Q-TOF mass spectrometer (QSTAR XL, Applied Biosystems) equipped with a nano-ESI source (Applied Biosystems) and a nano-ESI needle