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Activation of cyclin D1 affects mitochondrial mass following traumatic brain injury

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ABSTRACT

Cell cycle activation has been associated with varying types of neurological disorders including brain injury. Cyclin D1 is a critical modulator of cell cycle activation and upregulation of Cyclin D1 in neurons contributes to the pathology associated with traumatic brain injury (TBI). Mitochondrial mass is a critical factor to maintain the mitochondrial function, and it can be regulated by different signaling cascades and transcription factors including NRF1. However, the underlying mechanism of how TBI leads to impairment of mitochondrial mass following TBI remains obscure. Our results indicate that augmentation of CyclinD1 attenuates mitochondrial mass formation following TBI. To elucidate the molecular mechanism, we found that Cyclin D1 interacts with a transcription factor NRF1 in the nucleus and prevents NRF1's interaction with p300 in the pericontusional cortex following TBI. As a result, the acetylation level of NRF1 was decreased, and its transcriptional activity was attenuated. This event leads to a loss of mitochondrial mass in the pericontusional cortex following TBI. Intranasal delivery of Cyclin D1 RNAi immediately after TBI rescues transcriptional activation of NRF1 and recovers mitochondrial mass after TBI.

1. Introduction

Neurons are post-mitotic cells have permanently entered G0 phase and were incapable of entering the cell cycle (Frade and Ovejero-Benito, 2015). However, recent studies have shown that matured differentiated neurons can enter into the cell-cycle reentry phase which ultimately results in cell death rather than proliferation (Herrup and Yang, 2007; Kabadi et al., 2012; Kranenburg et al., 1996). The sequential activation of Ser/Thr kinases called the cyclin-dependent kinases (CDK), and their positive regulators (Kabadi and Faden, 2014; Malumbres, 2014) such as activation of Cyclin D1 are essential to maintaining the different stages of cell cycle. Dr. Faden and his associates have convincingly shown that cell cycle activation is responsible for cell death in apoptosis in the post-mitotic cells like neuron (Kabadi and Faden, 2014). In addition, activation of the cell cycle in microglial cells results in the release of pro-inflammatory and neurotoxic molecules which contributes significantly to the pathology of TBI (Byrnes and Faden, 2007; Byrnes et al., 2007; Di Giovanni et al., 2005; Hilton et al., 2008; Wu et al., 2011). However, its role in mitochondrial function is mostly unknown.

Mitochondrial dysfunction has been implicated in the TBI pathology (Fischer et al., 2016; Hill et al., 2017; Singh et al., 2006) and the maintenance of the mitochondrial mass has been suggested to function as a critical factor to maintain the mitochondrial function and production of ATP inside cells (Wang et al., 2017b). The proper intracellular distribution of mitochondria is assumed to be critical for normal physiology of neuronal cells (Cheng et al., 2012; Li et al., 2004). The changes in mitochondrial mass are correlated with the development and morphological plasticity of spines (Cheng et al., 2010; Li et al., 2004). Mitochondrial mass, by itself, represents the net balance between rates of biogenesis and degradation (Dominy and Puigserver, 2013) and mitochondrial mass can be regulated by mitochondrial DNA (mtDNA) content which is known to be synthesized inside the nucleus through activation of several transcription factors (Dominy and Puigserver, 2013; Jornayvaz and Shulman, 2010). PGC-1α is a co-transcriptional regulation factor that induces mitochondrial mass by activating different transcription factors, including NRF1, which promotes the expression of mitochondrial transcription factor A or TFAM (Dominy and Puigserver, 2013; Jornayvaz and Shulman, 2010; Scarpulla, 2011; Ventura-Clapier et al., 2008). NRF1 is an essential contributor to the sequence of events leading to the increase in transcription of key mitochondrial enzymes, and it has been shown to regulate TFAM, which drives transcription and replication of mtDNA (Jezierska-Drutel et al., 2013; Scarpulla, 2008). Previously it was shown that NRF1 could interact and acetylated by an acetyltransferase p300/CBP and acetylation of NRF1 enhances its transcriptional

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activation by augmenting its DNA binding (Izumi et al., 2003).

Here we have shown that mitochondrial mass was impaired following TBI due to the loss of mtDNA content. As a part of the mechanism, we found that activation of the cell cycle, notably, cyclin D1 directly contributes to the deficiency in mtDNA content by manipulating the transcriptional efficiency of NRF1.

2. Materials and methods

2.1. TBI procedure

The Committee on Animal Use for Research and Education at the University of Pittsburgh approved all animal studies, in compliance with National Institutes of Health guidelines. The procedure was done based on our previously published protocol (Farook et al., 2013; Kapoor et al., 2013; Sen et al., 2017). Briefly, 8–12-week old adult male C57BL/6 (Jackson Laboratory) mice were anesthetized with xylazine (8 mg/kg)/ketamine (60 mg/kg) and subjected to a sham injury or controlled cortical impact. Mice were placed in a stereotaxic frame (Ambient Instruments) and a 3.5 mm craniotomy was made in the right parietal bone midway between bregma and lambda with the medial edge lateral to the midline, leaving the dura intact. Mice were impacted at 4.5 m/s with a 20 ms dwell time and 1.2 mm depression using a 3-mm-diameter convex tip, mimicking a moderate TBI. Sham-operated mice underwent the identical surgical procedures but were not impacted. The incision was closed with 3 M Vetbond tissue adhesive and mice were allowed to recover in the heat pad. Body temperature was maintained at 37 °C using a small animal temperature controller throughout all procedures (Kopf Instruments).

2.2. Western blot and co-immunoprecipitation

Whole tissue lysates were prepared from 3 mm coronal sections centered upon the site of impact. A 1 mm micro punch was collected from the pericontusional cortex or the corresponding pericontusional hemisphere as described previously (Farook et al., 2013; Kapoor et al., 2013; Mir et al., 2014; Sen et al., 2017). Tissue was placed in RIPA buffer (containing protease and phosphatase inhibitor), sonicated, and centrifuged for 5 min at 12,000 × *g* at 4 °C. Fifty micrograms of protein were resolved on a 4–20% SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane. Blots were incubated overnight at 4 °C in primary antibody Cyclin D1, (Cell Signaling, 1:500 dilution), total NRF1 (Santa Cruz Biotech., 1:500 dilution), p300 (Sigma-Aldrich, 1:500 dilution), total PGC1α (Santa Cruz Biotech., 1:500 dilution), Nuclear NRF1 (Abcam, ab175932), nuclear PGC1α (Abcam ab54481), TFAM (Santa Cruz Biotech., 1:500 dilution), CoxII (Santa Cruz Biotech., 1:500 dilution), and Actin (Sigma-Aldrich, 1:5000 dilution) followed by a 2 h incubation with a Licor IRDye secondary antibody at room temperature. Blots were visualized using a Li-Cor Odyssey near-infrared imaging system, and densitometry analysis was performed using Quantity One software (Bio-Rad) (Farook et al., 2013; Kapoor et al., 2013; Mir et al., 2014). The intensity of each band was determined by ImageJ software, and the changes in the experimental band were represented as the fold change as described previously (Sen et al., 2017; Sen and Sen, 2016).

Protein-protein interactions and protein acetylation were measured by co-immunoprecipitation assay per our method (Farook et al., 2013; Kapoor et al., 2013; Mir et al., 2014; Sen et al., 2017). Briefly, treated or untreated cells were homogenized in lysis buffer containing 50 mm Tris, pH 7.4, 150 mM NaCl, 0.5% (v/v) tween-20, 50 mM Tris (pH 7.5), 1 mM EDTA with protease and phosphatase inhibitor by passing through 26-gauge syringe needle and centrifuged at 12,000 ×*g* for 5 min. 400μg of the total protein for each sample were incubated overnight with either CyclinD1 antibody (1:100), NRF1(1:100) or anti-IgG antibody for overnight. 30 μl of protein G agarose was added, and SDS-PAGE resolved co-immunoprecipitates and analyzed by western blotting with either the NRF1 antibody, p300 antibody or acetyl-lysine antibody.

2.3. Immunohistochemistry

Deeply anesthetized mice were perfused with phosphate buffer saline (0.1 M PBS, pH 7.4), followed by fixation with 4% paraformaldehyde in PBS. Brains were post-fixed for 1 h in 4% paraformaldehyde, followed by cryoprotection with 30% sucrose in PBS until brains permeated. Serial coronal sections of 20 μm were prepared using a cryostat microtome (Leica) and mounted directly onto glass slides before allowing them for drying for 1 h at room temperature. For immunofluorescence analysis sections were incubated 10 min at room temperature with 0.1% Triton X-100 in PBS containing, followed by overnight incubation at 4 °C with the primary antibody against CyclinD1 (1:100), NRF1 (1:100), PGC1α (1:100) or p300 (1:100 dilution). Sections were then washed with PBS and incubated with the appropriate Alexa Fluor-tagged secondary antibody for 2 h at room temperature in the dark. Sections were washed and mounted with sufficient drops of ProLong Gold Antifade Mountant with DAPI solution (Molecular Probes). Imaging was performed with the help of Olympus fluorescent microscope (IX83) and Nikon's C1 Digital Eclipse Modular Confocal Microscope Systems. The omission of primary antibody served as a negative control (Farook et al., 2013; Kapoor et al., 2013; Mir et al., 2014; Sen et al., 2017).

2.4. Intranasal delivery of cyclin D1-RNAi

CyclinD1-RNAi (Santa Cruz Biotechnology) were administered to 8–12 weeks C57BL/6 J mice through intranasal route using in vivo jetPEI (PolyPlus) transfection reagent as described previously with modifications (Bitko and Barik, 2008; Rodriguez et al., 2017). The RNAi-JetPEI complex was prepared according to the manufacturer's protocol with modifications (Aigner, 2006; Rodriguez et al., 2017). Briefly, either the cyclinD1 RNAi or control RNAi along with JetPEI were separately diluted into half the injection volume in a 10% sterile glucose solution where the final glucose concentration would have to be 5%. This formulation corresponds to nitrogen and phosphate (N/P) ratio of 7. Both the solutions were mixed by slight vortexing, and the JetPEI-RNAi mixture was incubated 15 min at room temperature. Intranasal administration of the Jet-PEI complex was performed 30 min after either sham or TBI surgery with the pipette tip to each nostril of the mouse. A 5μl of the jetPEI-RNAi complex was slowly administered to the nostrils maintaining a 2–3 s interval up to 10μl total/nostril of a mouse. After 5–10 s another 10μl of the solution was administered to the other nostril following the similar way for a total of 20μl/mouse and 10μg of siRNA/mouse. Mice were under observation for the entire solution disappears through the nasal cavity and till their consciousness. After 24 h all the mice were sacrificed, and brain samples were collected for further experiments.

2.5. Chromatin immunoprecipitation (ChIP) assay

For chromatin immunoprecipitation (ChIP) assays, we used a chromatin immunoprecipitation assay kit purchased from Millipore and followed the instructions from the supplier. ChIP assay was performed as described previously (Mir et al., 2014; Sen et al., 2017). Briefly, after sonication, tissue lysates containing soluble chromatin were incubated overnight with an anti-NRF1 antibody or with normal rabbit IgG. DNA–protein immunocomplexes were precipitated with protein A-agarose beads, washed, and eluted. The eluates were used as templates in PCR using the primers 5′-TTTGCTGTTGGGCA -3′ and 5′-CGGCGGC TTACCCCA -3′. The expected DNA fragment that was amplified is in the TFAM promoter region, which encompassed the NRF1 binding site.