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Research article

Pharmacological inhibition of spinal cord injury-stimulated ribosomal biogenesis does not affect locomotor outcome

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HIGHLIGHTS

- Ribosomal biogenesis is acutely upregulated after mouse contusive spinal cord injury.
- The upregulation coincided with spinal cord injury associated ER stress.
- New ribosomes may support cytotoxic recovery of translation after ER stress.
- However, inhibition of ribosomal biogenesis has minor effects on locomotor outcome.
- Hence, ribosomal biogenesis is not the critical cytotoxic effector of spinal cord injury.

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ABSTRACT

After unresolved endoplasmic reticulum stress, recovery of protein synthesis including increased expression of ribosomal components and translation factors may induce cell death. Using a mouse model of moderate contusive spinal cord injury (SCI) at the T9 level, upregulation of ribosomal biogenesis was observed in the injury epicenter at 24 h after trauma. Such upregulation coincided with endoplasmic reticulum stress response as previously reported in this model. It was also accompanied by changes in expression of many other genes associated with translational regulation. Systemic treatment with a pharmacological inhibitor of RNA-Polymerase-1, BMH-21 reduced rRNA transcription in the spinal cord. Moreover, in the injury epicenter, treatment with BMH-21 increased expression of oligodendrocyte-specific transcripts including *Mbp* and *Cldn11* at 3 days post injury. Although such findings may suggest at least transient reduction of oligodendrocyte death, locomotor outcome was mostly unaffected except slightly accelerated recovery of hindlimb function at week 2 post-injury. Therefore, at least in mice, RNA-Polymerase-1 does not appear to be a robust target for therapies to protect spinal cord tissue after contusion. However, these findings raise an interesting possibility that altered rate of ribosomal biogenesis contributes to the apparent translational reprogramming after contusive SCI. Such a reprogramming could be a major regulator of SCI-induced gene expression.

1. Introduction

Impairment of endoplasmic reticulum (ER) function leads to ER stress. Cells respond to such a challenge with the conserved ER stress response (ERSR) whose primary goal is to restore ER homeostasis. However, if that is not possible, the pro-apoptotic arm of the ERSR induces apoptosis. Recent work documented that the ERSR is activated after SCI and that its genetic or pharmacological modulation protects white matter, prevents oligodendrocyte apoptosis and improves functional recovery after trauma [20,21].

Abbreviations: Atf4, activating transcription factor 4; BMS, basso mouse scale; Chop, CCAAT-enhancer-binding protein homologous protein; EAE, experimental autoimmune encephalomyelitis; ERSR, endoplasmic reticulum stress response; GO, gene ontology; MS, multiple sclerosis; mTOR, mechanistic target of rapamycin; Npm1, nucleophosmin-1; OPC, oligodendrocyte precursor cell; Pol1, RNA-Polymersase-1; qRT-PCR, quantitative reverse transcriptase PCR; SCI, spinal cord injury.

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The transcription factors CCAAT-enhancer-binding protein homologous protein (Chop/Ddit3) and activating transcription factor 4 (Atf4) are recognized as principal mediators of the ERSR-induced cell death. Recent work has identified an unorthodox mechanism by which Chop and Atf4 kill cells with a dysfunctional ER [13]. While global inhibition of protein synthesis is the early response to ER stress, relieving the ER from excessive protein overload, the subsequent activation of Atf4 and Chop increases global protein synthesis. If ER homeostasis has not been yet restored, the resulting protein overload of the ER induces oxidative stress, mitochondrial damage and cell death. Mechanisms that were implicated in such a toxic wave of translation include increased amino acid import, enhanced tRNA synthetase activity, activation of mTOR, and an increased supply of ribosomes [12,17,13].

Protein synthesis is carried out by ribosomes whose cellular content appears to limit cellular capacity of translation [7,9]. Thus, in many situations that require increased protein synthesis, like proliferation or cell hypertrophy, ribosome production is enhanced [5,7,9]. Intriguingly, many ribosomal biogenesis-associated genes are up-regulated by Atf4 and/or Chop, suggesting that increased ribosome supply contributes to the translational catastrophe after lethal ER stress [13]. Indeed, the potentially deleterious role of ribosomal biogenesis in ER stress-exposed cells has been documented in yeast [24].

The RNA polymerase I (Pol1)-driven transcription of ribosomal RNA (rRNA) initiates ribosome synthesis [15]. Pol1 is the major regulator of ribosome production. As cancer cells hijack ribosome biogenesis to fuel their growth, Pol1 became a target of novel anticancer drugs including the clinically tested CX-5461 and CX-3543 or the recently identified BMH-21 [6,8,10,23]. If applied transiently, such agents kill cancer, but not normal, cells. While Pol1 activity is reduced immediately after induction of ER stress, ribosome synthesis resumes at later time points with many ribosomal component genes being upregulated by Atf4/Chop [11,13]. Although cells with reduced capacity of ribosome synthesis are more resistant to ER stress [13,24], emerging pharmacological inhibitors of Pol1 have not been tested for their therapeutic potential in ER stress diseases such as SCI. The current study was initiated to examine whether ribosomal biogenesis is regulated after SCI and whether Pol1 inhibitors may affect SCI outcome.

2. Methods

Animals. Adult (8–10 weeks old) C57Bl/6 female mice were obtained from Harlan (Indianapolis, IN) and gentled for 5–7 days before experiments. All animal experiments strictly followed a protocol that was approved by University of Louisville Institutional Animal Care and Use Committee.

Spinal cord injury was performed as described previously [21]. Briefly, animals were anaesthetized by an *i.p.* injection of 250 mg/kg body weight avertin. Gentamycin (50 mg/kg; Boehringer Ingelheim, St. Joseph, MO) was administered subcutaneously to reduce infection. Moderate contusion injuries (50 kdyn force/400–600 μm displacement) were performed using the IH impactor (Infinite Horizons, Lexington, KY) following a laminectomy at the T9 vertebrae. In SCI studies with BMH-21, comparison of actual injury force readings and tissue displacements revealed no significant differences between experimental groups (supplementary Table S1). Therefore, similar injury severity was consistently obtained using such a methodology. In the SCI pre-rRNA analysis experiment, controls included sham animals that received only a T9 laminectomy.

RNA extraction and analysis was accomplished using standard techniques [21,16]. Briefly, total RNA was be extracted from spinal cord tissue at the injury epicenter (a 3 mm-long segment spanning the injury site) using Trizol (Invitrogen, Carlsbad, CA). Following cDNA synthesis with random hexamers qRT-PCR was performed using Syber-Green DNA dye (pre-rRNA/18S rRNA) or Taq-man universal PCR master mix (*Mbp*, *Cldn11*, *Gfap*, *Gapdh*). The following primers were used: pre-rRNA forward- ctcctctctcgcgctctctgtc, reverse- gcatggcttaatctttgagacaagca; 18S rRNA forward- gttggttttcggaactgaggc, reverse- gtcggcatcgtttatggtcg; Assay on Demand primers for *Mbp*, *Claudin11*, *Gfap* and *Gapdh* were described previously [21]. RNA levels were quantified using the ΔΔCT method; reference genes were as indicated.

BMH-21 treatment. BMH-21 was dissolved in 20 mM citrate buffer (pH 6.0) and administered by intraperitoneal injections (0.1–0.2 ml/injection) as indicated. Vehicle controls were also included. Detailed experimental design of SCI studies with BMH-21 is presented in supplementary Table S1.

Oligodendrocyte Precursor Cell (OPC) culture. Adult rat spinal cord OPCs were prepared and cultured as described [26]. Their survival was monitored by MTT assay following a standard protocol [14].

Nucleophosmin–1 (Npm1/B23) immunofluorescence was performed using a mouse monoclonal antibody (Sigma) as previously reported [16].

Locomotor function was evaluated as previously reported [21]. Briefly, Open field Basso Mouse Scale (BMS)] locomotor analyses [3] were performed at baseline scores and weekly following SCI for 5–6 weeks. All raters were trained by Dr. Basso and colleagues at the Ohio State University and were blinded to animal groups.

Meta-analysis of RNA microarray data. Publicly available data from Affymetrix RNA microarray analysis of injury epicenter region after moderate contusive SCI (A. Faden, NCBI's GEO GSE5296, all experiments in C57Bl6 female mice) were accessed and lists of 2167 (4 h post-SCI) or 2819 (24 h) or 4652 (72 h post SCI, data not shown) significantly affected genes ($p < 0.01$) were retrieved by comparing injury epicenter regions ($n = 3$) to sham controls ($n = 4$ including 2 controls from the same time point as SCI + 2 additional controls from the closest time point to that of SCI; such strategy was used as only 2 sham controls were available for each time point). NCBI's DAVID was used to analyze gene ontology term (GO) enrichment among affected genes. FDR (q value) was use as a primary measure of GO enrichment.

Statistical analysis. Result of qRT-PCR and MTT survival assay were analyzed using the non-parametric *u*-test as limited n number (3–4/group) precluded normality testing. BMS data were analyzed by repeated measure ANOVA and Tukey *post-hoc* tests; one-way ANOVA was used to analyze SCI force and tissue displacement data.

3. Results

Our prior work has documented contusive SCI-associated ER stress including increased mRNA expression of cytotoxic transcription factors *Atf4* and *Chop* in the injury epicenter at 6- and 24 h after contusion [21]. We used the same total RNA material to investigate SCI effects on expression of pre-rRNA. Due to relatively rapid processing, pre-rRNA is a good indicator of Pol1 activity which, by transcribing rRNA genes, mediates the critical step of ribosomal biogenesis [9]. After SCI, higher pre-rRNA levels were observed at 24- and 72 h post injury (Fig. 1A). The maximal increase of 1.4-fold sham controls was seen 24 h post injury. Such a dynamic pattern fits a model of ribosomal biogenesis being under Atf4/Chop-dependent regulation following SCI.

To further explore SCI effects on ribosomal biogenesis, meta-analysis of publicly available data from RNA microarray studies of mouse contusive SCI was performed. A data set from Dr. Alan Faden's laboratory was chosen as it was obtained from similar animals (C57Bl6 mice, moderate contusion at the T8 level) and covered similar/identical time points to those of our study (accession number GSE5296) [22]. Thus, gene ontology (GO) analysis of sig-