

Therapeutic Treatment for Regulation of RhoA Pathway in Intervertebral Disc Degeneration

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Disclosures: The authors have no conflicts of interest to disclose

INTRODUCTION: Low back pain is a leading cause of disability in the world and is linked with intervertebral disc (IVD) degeneration, which involves ECM degradation, structural defects, and loss of IVD height. Pro-inflammatory cytokines are key mediators of IVD degeneration^{1,2}. Prior studies by our group have shown that RhoA can regulate the pro-inflammatory transcription factor NF- κ B and its downstream signaling and degradative effects on the IVD³. We also showed that stimulation of cells with TNF α *in vitro* leads to dysregulation in F-actin, decreased phosphorylated myosin light chain (pMLC) an activator of contractile myosin II, and decreased expression of RhoA and myosin II genes (Myl6, Myl9, Myh9)³. However, the extent to which RhoA expression and actomyosin contractility is disrupted in IVD degeneration *in vivo* is unknown. Targeting RhoA GTPase signaling may be an attractive approach to regulating actomyosin contractility and downstream degradation of the ECM. The goal of this study is to evaluate changes in RhoA in an *in vivo* rat needle puncture IVD model and to develop a treatment approach for promoting RhoA activity. We utilized a small molecule RhoA activator drug, CN03, which blocks GAP stimulated and intrinsic GTPase activity⁴. To begin to understand the effectiveness of CN03 as a therapeutic, we evaluated its effects on bovine NP cells *in vitro* in the presence or absence of a specific RhoA inhibitor (CT04). We hypothesize that IVD puncture will decrease RhoA expression and activity, which can be recovered via treatment with CN03 administration.

METHODS: Caudal Needle Puncture Injury: This study received IACUC approval. Under a fluoroscopic guidance, a 20G needle was used to puncture the caudal IVDs of male Sprague Dawley rats. The needle was inserted halfway into the IVD, rotated 360° and held in place for 30 seconds (n=6). Caudal IVD (same levels) from uninjured rats were used as controls (n=6). IVDs were dissected at 14- and 28-days post injury and the NP was separated out and snap frozen. NP was pulverized using a bead homogenizer and RNA was isolated using TRIzol and high salt solution. **Bovine NP cell isolation and culture:** NP tissue was dissected from multiple levels of a lumbar spine from a juvenile cow, pooled, and digested in complete media [high glucose Dulbecco's modified Eagle's medium (DMEM) + 10% fetal bovine serum (FBS) + 1% penicillin-streptomycin (PS) solution] supplemented with collagenase type I (0.3 mg/ml; Sigma-Aldrich) and collagenase type II (0.3 mg/ml; Sigma-Aldrich) for 3 hours at 37°C with gentle agitation. Bovine NP (bNP) cell digest was passed through a 70- μ m cell strainer, washed, counted, and grown in complete medium. For treatment, P2 cells were plated in a 12-well plate at 200K cells/well. **Treatments:** Cells were treated for 24 hours in the following groups: (i) untreated, (ii) CN03 (0.2, 0.5, 1, or 5 μ g/ml, Rho activator, Cytoskeleton Inc), (iii) CT04 (1.5 μ g/ml, Rho inhibitor, Cytoskeleton Inc) + CN03 (0.2, 0.5, 1, or 5 μ g/ml). In group (iii) the cells were pretreated with CT04 for 2 hours before treatment for 24 hours with CN03. In some groups, treated cells were lysed for RNA isolation with TRIzol and RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. **RhoA Activation Assay:** Active RhoA levels were measured using the RhoA G-LISA Activation Assay kit (BK124, Cytoskeleton Inc) and normalized to total RhoA levels, which were measured using the Total RhoA ELISA kit (BK150, Cytoskeleton Inc) according to the manufacturer's protocol. **Gene Expression:** RT-qPCR was conducted to measure expression of RhoA and myosin II genes (Myl6, Myl9, Myh9, Myh10).

RESULTS: At 14 days post-injury, Myl6 gene expression was significantly lower in injured IVDs vs. control. By day 28 post-injury, both the expression of RhoA and Myl6 significantly decreased in injured IVDs vs. control (Figure 1). Stimulation of bovine NP cells with CN03 increased levels of Myl6, Myl9 and Myh9 with 5 μ g/ml dose compared to untreated, however no significant change in RhoA or Myh10 gene expression was observed (Figure 2). However, CN03 stimulation of bNP cells after 24 hours resulted in significant increases in active RhoA levels in all doses compared to untreated (Figure 3a). Following inhibition of RhoA with CT04, all doses of CN03 doses were also able to recover active RhoA levels to above that of control levels (Figure 3b).

DISCUSSION: The goal of this study was to evaluate RhoA expression levels in IVD degeneration *in vivo* and to begin evaluating the potential therapeutic benefit of using CN03 as a treatment to increase RhoA activity and related actomyosin contractility. Puncture injury to the IVD decreased expression of RhoA and Myl6, which is consistent with changes in bNP cells due to TNF α stimulation³ and changes in human IVD degeneration⁵. Treatment of bNP cells with CN03 promoted increases in active RhoA levels and increased expression of myosin II genes Myl6, Myl9 and Myh9. Moreover, CN03 also significantly increased active RhoA levels following direct inhibition with CT04. These findings demonstrate that CN03 is effective at re-activating RhoA after its pathway is disrupted, suggesting that CN03 may be effective at reactivation of RhoA signaling in IVD inflammation or degeneration. Future studies will examine the therapeutic effects of CN03 *ex vivo* IVD organ culture model and *in vivo* puncture models and investigate the potential for drug delivery of CN03 *in vivo*.

SIGNIFICANCE/CLINICAL RELEVANCE: Actomyosin contractility mediated by RhoA GTPase presents a novel therapeutic target to prevent pro-inflammatory cytokine induced degradation of the IVD ECM.

REFERENCES: [1] An, HS. Et al, 2004, Spine [2] Risbud, M. V. et al, 2014, Nature Reviews [3] Hernandez, P. A., et al, 2020, Science Advances [4] Flatau, G. et al, 1997, Nature [5] Babu, N. et al, 2016, The Spine Journal

ACKNOWLEDGEMENTS: Supported in part by NIH NIAMS R21 AR080516, R01 AR077760, T32 AR080744.

FIGURES:

