TLR4 Inhibitor Prevents Biophysical Changes in Nucleus Pulposus Mechanobiology Induced by Inflammatory Stimulation

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Introduction: Intervertebral disc (IVD) degeneration (DD) is characterized by increased catabolic activity, ECM breakdown, and elevated levels of pro-inflammatory cytokines, particularly TNF-α and IL-1β [1]. DD also modulates the hydrostatic pressure and biomechanical loading in the nucleus pulposus (NP) [2,3], potentially leading to altered biomechanical microenvironment around the NP cells. In a previous study we have shown that stimulation of isolated NP cells with the inflammatory cytokine TNFα, or with lipopolysaccharide (LPS), a ligand of the toll-like-receptor-4 (TLR4) inflammatory pathway, causes significant increases in both cell volume and hydraulic permeability of the cell [4]. Our findings indicated that inflammatory stimulation can significantly alter NP mechanobiology, potentially providing a link between disease etiology and mechano-responsiveness of NP cells. In the current study, we examined the effect of TLR4 inhibition on mitigation of inflammatory induced biophysical changes of NP cells using TAK-242, a small molecule inhibitor of TLR4. TAK-242 is a cell-permeable cyclohexenecarboxylate that disrupts TLR4, but not TLR1-3 or TLR5-10, through interaction with intracellular adaptor molecules TIRAP and TRAM [5]. We hypothesize that disruption of the TLR4 pathway by TAK-242 will prevent changes in mechanobiology of NP cells induced by inflammatory stimulation with LPS or TNFα.

Methods: NP Cell Isolation: NP tissue was isolated from bovine lumbar spines, minced and digested using standard enzymatic protocols. Cells were cultured for 1-2 passages in DMEM +10% FBS + 1% AA. Inflammatory Challenge and TLR4 Inhibition: After culturing in 24-well plates overnight, FBS containing media was removed and replaced with serum-free media supplemented with either LPS (0.1 µg/ml) or TNFα (10ng/ml) for 24 hours. For TLR4 inhibition groups, cells were pre-treated in serum-free media supplemented with TAK-242 (1µM, 10µM, 100µM) for 1 hour prior to addition of LPS or TNFα. For osmotic loading experiments, TLR4 inhibition groups were treated with 100µM TAK-242. NO Assay: Supernatants were collected and nitrite (NO) concentration was measured with Griess reaction. LDH Assay: Supernatants were also analyzed for lactate dehydrogenase (LDH, an indicator of cell death) using Roche Cytotoxicity Detection Kit. Gene Expression: RNA was isolated and pooled from treated cells and gene expression of IL-6 and GAPDH were analyzed using qRT-PCR. Osmotic Loading and Imaging: NP cells were seeded in a custom made Y-shaped microfluidic channel [4,6,7]. Cells were equilibrated in a 333 mOsm/L NaCl solution after which a single hyper-osmotic loading followed by a hypo-osmotic loading step was applied with NaCl solutions at 466mOsm/L followed by 333 mOsm/L. During osmotic loading, cells were imaged using differential interference contrast (DIC) at 0.5 Hz. A custom made matlab routine was used to segment individual cells and calculate a volume response over time for each cell. Volume response was curve fitted to a mixture theory framework in order to determine biophysical properties for each cell including membrane hydraulic permeability (Lp), and reference intracellular water content (Φir) [4,7]. Data was analyzed with ANOVA and Fisher LSD post-hoc test, with p <0.05 considered significant.
**Results:** TAK-242 significantly inhibited LPS and TNF induced nitrite release in a dose dependent manner, with complete nitrite inhibition observed between 10 and 100µM dose (Figure 1A,C). IL-6 gene expression was also down regulated in a dose-dependent manner due to TAK-242 treatment, in both LPS and TNFα stimulated cells (Figure 1B,D). No differences in cell viability were observed between groups. Both LPS and TNFα stimulation was found to lead to significant increases in Lp for both osmotic steps (Figure 2A). Treatment of cells with TAK-242 significantly reduced Lp back to baseline levels in both LPS and TNFα groups (Figure 2A). No significant changes in Φir were observed due to inflammatory simulation. Though cells in the LPS + TAK group exhibited a significantly higher Φir compared to control LPS or control.

**Discussion:** The goal of this study was to investigate the effects of TLR4 inhibition on inflammatory mediators and the biophysical properties of isolated NP cells. TAK-242, a small molecule TLR4 inhibitor, was found to decrease LPS and TNF-α induced NO release in a dose dependant manner. Complete inhibition of NO release and IL-6 upregulation confirms that TAK-242 is able to prevent LPS and TNFα induced inflammation in NP cells. The effects of TAK-242 (at 100 µM) was further evaluated in osmotic loading experiments. LPS and TNFα were found to increase Lp in NP cells, consistent with our previous study [4]. Interestingly, treatment of cells with TAK-242 inhibited LPS and TNFα induced changes in Lp, where Lp was found to be comparable to baseline control levels. These findings indicate that TAK-242 may prevent alterations in biophysical properties and protect the mechanobiological function of NP cells from inflammatory induced changes. Interestingly, TAK-242 was equally effective at mitigating responses by TNF and LPS stimulation, suggesting that TNFα may modulate the response of NP cells via a link to the TLR4 pathway.

**Significance:** This study has demonstrated that inhibition of TLR4 can mitigate changes in mechanobiological properties of NP cells. TLR4 plays an important role in the response of disc cells to inflammation and subsequent disc degeneration. Consequently, our findings identify a potential target and therapeutic agent for further consideration in the treatment of disc degeneration.
Figure 1: TAK-242 elicits a complete, dose-dependent inhibition of nitrite release in NP cells stimulated with LPS [A] or TNF-α [C]. *p<0.05 relative to positive control (no inhibitor). [B, D] IL-6 expression is downregulated in a dose-dependent manner with TAK-242 treatment in both LPS and TNF stimulation.

Figure 2: Biophysical properties during hypo-osmotic loading step (468-333 mOsm NaCl). [A] Stimulation with LPS or TNF significantly increased Lp. TAK-242 treatment significantly decreased Lp back to baseline levels. [B] LPS + TAK-242 group exhibited significant higher $\Phi_p$ versus control and LPS alone. *p<0.05 versus control; ^p<0.05 versus respective TAK group.

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