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Inhibition of Fibroblast Activation to the Myofibroblast Phenotype Using a Small Molecule Aldehyde Trap

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INTRODUCTION

Fibroblast activation leads to the formation of myofibroblasts, which cause fibrosis and scarring in injured tissues. Activation occurs when injury leads to lipid peroxidation and activation of downstream pro-inflammatory signaling pathways. Studies have suggested that cellular aldehydes, formed as byproducts of lipid peroxidation, play a role in this inflammatory response to injury. ADX-102 is a small molecule aldehyde trap that covalently binds and sequesters cellular aldehydes, including malondialdehyde and 4-hydroxynonenal. Treatment of cardiac fibroblasts with ADX-102 inhibited transformation to the myofibroblast phenotype both in untreated and H_2O_2-treated cells. ADX-102 inhibited translocation of NFκB, and decreased production of IL-1β, suggesting that ADX-102 limited activation of this pro-inflammatory pathway. These data indicate that ADX-102 has anti-inflammatory properties that limit the activation of myofibroblasts and therefore may limit fibrosis following injury.

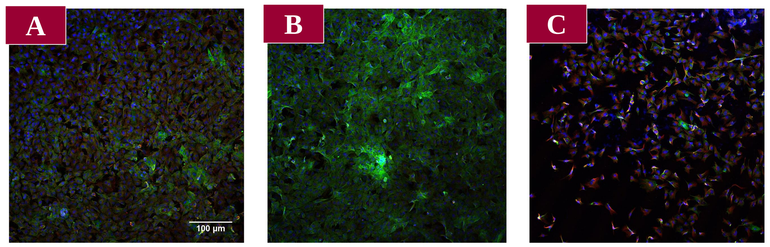
METHODS

After isolation, neonatal cardiac fibroblasts were allowed to adhere for 2 hours in serum-free DMEM. Non-adherent cells were then removed and adherent cells were fed with DMEM + 10% FBS. Cells were maintained for 24 hours before treatments. In initial experiments, fibroblasts were treated with H_2O_2 to induce transformation to the myofibroblast phenotype. H_2O_2 was omitted in later experiments, as cells auto-activated over the first 24 hours in culture. Cells were then incubated for 24 hours in the absence or presence of increasing concentrations of ADX-102. Following incubation, cells were rinsed in PBS and prepared for immunostaining and Western blot analysis. For immunostaining, cells were incubated with primary antibodies overnight at 4˚C, then with secondary antibodies conjugated to a fluorophore for 1 hour, and finally incubated with DAPI to stain nuclei. Cells were and viewed using a Leica SP8 confocal microscope equipped with a 10X air lens. For Western blotting, cells were lysed in RIPA buffer, run on 8% gels, transferred to nitrocellulose and stained with primary and secondary antibodies before ECL detection.

SUMMARY/CONCLUSIONS

ADX-102 inhibits fibroblast to myofibroblast transformation in cultured cardiac fibroblasts via the NFκB pathway, by limiting NFκB nuclear translocation and subsequent expression of pro-inflammatory cytokines, such as IL-1β. This suggests that aldehyde traps may represent a novel therapeutic class, able to limit inflammation and fibrosis following tissue injury.

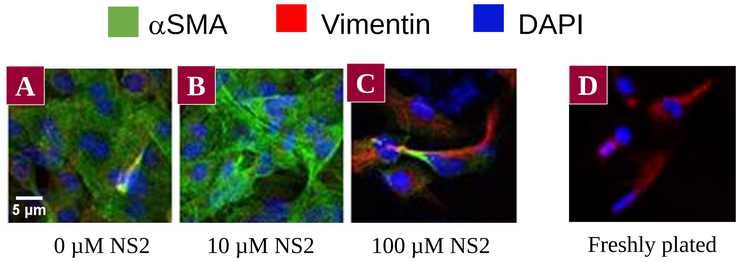
RESULTS



0 µM ADX-102 | 10 µM ADX-102 | 100 µM ADX-102

Figure 1: **ADX-102 blocks phenotypic changes associated with the fibroblast to myofibroblast transformation**. α-SMA and vimentin immunostaining of cardiac fibroblasts. A In the absence of ADX-102, fibroblasts showed high levels of α-SMA and a flattened morphology, indicative of fibroblast transformation to myofibroblasts. B Treatment with 10 µM ADX-102 showed no significant change in α-SMA or myofibroblast morphology. C Treatment with 100 µM ADX-102 resulted in a significant decrease in α-SMA expression and reversion to the quiescent, non-activated fibroblast morphology.

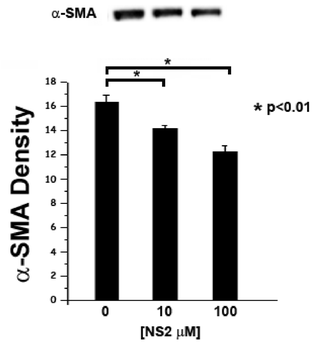
α-SMA: α-smooth muscle actin, a marker of transformation to the myofibroblast phenotype. Vimentin: a marker of the untransformed fibroblast phenotype



0 µM NS2 | 10 µM NS2 | 100 µM NS2 | Freshly plated

Figure 2: **ADX-102 limits α-SMA expression and transformation to the motile morphology associated with activated myofibroblasts (high magnification of cells in Figure 1)** A, B In the absence of ADX-102 or in the presence of a low concentration (10 µM) of ADX-102, cells show the highly flattened morphology of an activated myofibroblast. C Cells treated with 100 µM ADX-102 do not exhibit signs of transformation, and show the elongated morphology of a freshly plated fibroblast (D), prior to activation.

Figure 3: **ADX-102 treatment results in lower levels of α-SMA levels in cultured fibroblasts.** Western blot analysis showed that in the absence of ADX-102, activated fibroblasts produce high levels of α-SMA. Treatment with 10 µM or 100 µM ADX-102 significantly decreased levels of α-SMA, confirming the observations made by confocal microscopy, and indicating that ADX-102 inhibits activation of fibroblasts to the activated myofibroblast phenotype.



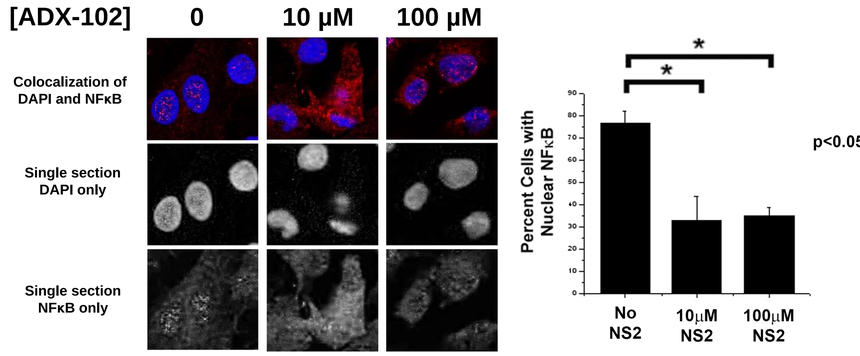


Figure 4: **ADX-102 inhibits translocation of NFκB to the nucleus**. As NFκB translocation to the nucleus is essential for the transcription of pro-inflammatory mediators, NFkB cellular localization was examined by confocal microscopy following incubation in the presence or absence of ADX-102. After staining, cells were counted and the percent of cells with NFκB in the nucleus were determined. Nuclear NFκB was observed in the majority (76.6%) of cells cultured in the absence of ADX-102, whereas cells treated with 10µM or 100µM ADX-102 showed significantly fewer cells with nuclear NFκB (30.7% and 35.7% respectively).

Figure 5: **ADX-102 treatment results in decreased levels of NFκB**. Western blot analysis showed that treatment of activated fibroblasts with 10µM or 100µM NS2 significantly decreased total NFκB in these cells, suggesting that ADX-102 can downregulate a critical protein in a pro-inflammatory pathway.

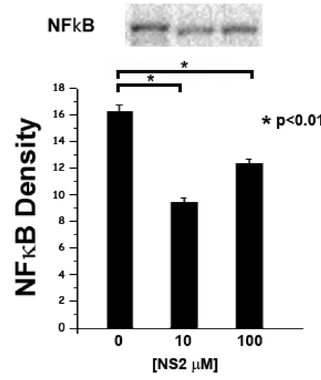
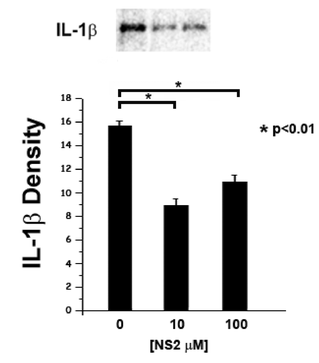


Figure 6: **ADX-102 treatment results in decreased levels of IL-1β**. Inhibition of NFκB nuclear translocation may be expected to inhibit upregulation of pro-inflammatory cytokines, such as IL-1β, which has been shown to be upregulated in other systems in which fibroblasts have been activated to myofibroblasts. In this system, Western blot analysis showed that activated fibroblasts in the absence of ADX-102 showed high levels of IL-1β, which were significantly decreased in the presence of both 10 µM and 100 µM ADX-102.



ADX-102 = NS2