Rho GTPases are Required for Smad3-Mediated Induction of ET-1 in Human Trabecular Meshwork Cells

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INTRODUCTION

Primary open-angle glaucoma (POAG) is a leading cause of irreversible blindness, affecting approximately 2.7% of adults 40 years of age and older in the US. A poorly understood risk factor for the initiation and progression of POAG is elevated intraocular pressure (IOP). In healthy eyes, normal IOP levels (10-21 mmHg) are maintained through balanced aqueous humour production and outflow of aqueous humour (AH). In adults, the majority (>90%) of AH flows by way through the conventional outflow pathway, beginning with the trabecular meshwork (TM). Resistance to AH outflow through the TM is mediated, in part, through Rho GTPases (RhoA and RhoB), which are downstream effectors of Smad3 in the TGF-β signaling pathway. In this study, we examine the role of Smad3 in the regulation of ET-1 expression and secretion in human trabecular meshwork cells.

RESULTS

**Figure 1. TGF-β induces ET-1 expression and secretion through TGFβRI/ALK-5 signaling**

Human TM cells were pre-treated (24h) without or with SB-431452 (1 µM) or with SB-515342 (1 µM) in the absence (200 mM HCl) or presence of TGF-β2 (5 ng/ml). ET-1 expression and secretion was quantified by ELISA. Data shown are the mean ± SD (n=3) from 3 separate experiments. *p < 0.01 compared to vehicle, one-way ANOVA with Dunnet’s post-hoc analysis.

**Figure 2. Smad3 is required to facilitate TGF-β2 mediated ET-1 expression and secretion**

**A.** Western Blot: Inhibitors of phosphorylated Smad3 (pSmad3) and total Smad3 proteins were resolved by western blotting using rabbit antibodies directed against Smad3 (25 nM), RhoA (100 nM), and GAPDH. (20-30 µg per lane) were resolved as we have previously described (Von Zee et al., 2012). Proteins were visualized by enhanced chemiluminescence. **B.** Content of secreted ET-1 peptide present in culture medium was quantified by ELISA. Data shown are the mean ± SD (n=3) from 3 separate experiments. *p < 0.01 compared to vehicle, one-way ANOVA with Dunnet’s post-hoc analysis.

**Figure 3. Rho subfamily GTPases facilitate TGF-β2 mediated ET-1 expression and secretion**

**A.** Western Blot: Primary human TM cells were pre-treated (24h) without (0.05% glycerol) or with C3 (10 µM) and incubated (24h) in the absence (200 mM HCl) or presence of TGF-β2 (5 ng/ml) as indicated. (Top) Relative content of pET-1 mRNA was quantified by qRT-PCR and normalized to GAPDH. (Bottom) Content of secreted ET-1 peptide present in culture medium was quantified by ELISA. Data shown are the mean ± SD (n=3) from 3 separate experiments. τp<0.01 compared to vehicle, one-way ANOVA with Dunnet’s post-hoc analysis.

**Figure 4. RhoA GTPases promote TGF-β2 mediated induction of ET-1**

**A.** Western Blot: Transformation of Human TM cells were pre-treated (24h) without (0.05% glycerol) or with Y-27632 (10 µM) or with vehicle (DMSO 0.2% v/v) as indicated and incubated (24h) in the absence (200 mM HCl) or presence of TGF-β2 (5 ng/ml) as indicated. (Top) Relative content of pET-1 mRNA was quantified by qRT-PCR and normalized to GAPDH. (Bottom) Content of secreted ET-1 peptide present in culture medium was quantified by ELISA. Data shown are the mean ± SD (n=3) from 3 separate experiments. *p < 0.01 compared to vehicle, one-way ANOVA with Bonferroni’s post-hoc analysis.

**Figure 5. Inhibiting Rho GTPase does not alter TGF-β2 mediated Smad3 phosphorylation**

Primary human TM cells were pre-treated (24h) without (0.05% glycerol) or with C3 (10 µM) and incubated (24h) in the absence (200 mM HCl) or presence of TGF-β2 (5 ng/ml) as indicated. Shown is a representative Western immunoblot of phosphorylated and total Smad3 proteins. Data are representative of 2 separate experiments performed in duplicate.

**Figure 6. TGF-β2 mediated ET-1 induction occurs independently of Rho kinase signaling**

Transformed human TM cells were pre-treated (24h) without (0.05% ethanol) or with Y-27632 (10 µM) as indicated and incubated (24h) in the absence (200 mM HCl) or presence of TGF-β2 (5 ng/ml) as indicated. (Top) Relative content of pET-1 mRNA was quantified by qRT-PCR and normalized to GAPDH. (Bottom) Content of secreted ET-1 peptide present in culture medium was quantified by ELISA. Data shown are the mean ± SD (n=3) from 1 experiment. *p < 0.01 compared to vehicle, one-way ANOVA with Bonferroni’s post-hoc analysis.

**Figure 7. TGF-β2 increases outflow resistance in perfused porcine anterior segments**

Pressure measurements were performed in ex-vivo isolated perfused porcine anterior segments using a Harvard Apparatus pressure transducer calibrated in-house. Data are representative of 1 experiment.

CONCLUSION

TGF-β2-mediated increases in IOP may occur, in part, through induction of Smad3- and RhoA-dependent ET-1 synthesis and secretion from human TM cells.

METHODS

Cell Culture: Primary human TM cells were harvested and purified from extracted human cadaveric (n=6) or we have previously described (Von Zee et al., 2012). To pharmacologically inhibit TGFβRI/ALK5 signaling, the following inhibitors were used: SB-431452 (1 µM) or SB-515342 (1 µM) to inhibit TGFβRI/ALK5 activator, SB-431223 (100 nM) or SB-517172 (100 nM) to inhibit TGFβRII, a specific inhibitor of RhoA and C3 (10 µM) a specific inhibitor of both RhoA and RhoB. cell culture medium was maintained at 37°C in an atmosphere of 5% CO₂/95% air. Anterior segment perfusion are performed on-site at Hines VA Hospital using fresh porcine eyes obtained from a local vendor. Experiments were performed on 1-2 experiments. *p < 0.01; **p < 0.001 compared to vehicle, one-way ANOVA with Bonferroni’s post-hoc analysis.

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