TGF-β2 Attenuates Constitutive Expression of Bradykinin B2 Receptors in Human Trabecular Meshwork Cells

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Jonathan D. Lautz[1,2]; Cynthia L. Pervan[2,3]; Kelly A. Langert[2] and Evan B. Stubbs, Jr.[1,2,3]

[1] *Program in Neuroscience, Loyola University Chicago, Maywood, IL, United States*

[2] *Research Service (151), Edward Hines Jr. VA Hospital, Hines, IL, United States*

[3] *Department of Ophthalmology, Loyola University Chicago, Maywood, IL, United States*

LOYOLA MEDICINE — Loyola University Chicago Stritch School of Medicine

INTRODUCTION

Glaucoma is a leading cause of blindness, projected to affect nearly 80 million people worldwide by the year 2020.[1] In the US, it is estimated that nearly 2 million individuals age 45 years and older have primary open angle glaucoma (POAG), the most prevalent form of the disease.[2] Current treatment options for patients with POAG are aimed at lowering chronically elevated intraocular pressure (IOP), a poorly-understood risk factor associated with POAG.[3]

In healthy eyes, normal IOP is maintained through balanced production and outflow of aqueous humor (AH). In adults, the majority (>50%) of AH exits the eye through the trabecular meshwork (TM).[4] Increased resistance to AH outflow through the TM is considered to be a significant contributor to aberrant elevation of IOP in POAG patients. *The mechanism by which this occurs remains poorly defined.*

In the anterior chamber of POAG patients, the levels of transforming growth factor (TGF)-β2 in AH is aberrantly elevated compared to healthy eyes.[5-7] Similarly, perfusion of TGF-β2 through cultured human, bovine, and porcine anterior segments significantly elevates IOP *ex vivo* by increasing outflow resistance through the TM.[8-9] *In vitro,* exogenous addition of TGF-β2 has been shown to increase content of a number of factors associated with elevated IOP, including extracellular matrix components as well as endothelin-1.[9-10] *However, there remains a paucity of data on the complete mechanism by which TGF-β2 promotes aberrantly elevated IOP.*

Activation of the bradykinin (BK) receptor B2 has recently been shown to lower IOP in ocular hypertensive non-human primates.[11-12] By comparison, TGF-β2 is known to modulate BK-associated signaling pathways.[13-14] However, the relationship between TGF-β2 and BK signaling in TM cells remains undefined. *In this study, we investigated the effects of TGF-β2 signaling on IOP and B2 receptor content in porcine anterior segments and human TM cells, respectively.*

METHODS

Cell Culture: Primary human TM (hTM) cells were harvested from discarded human corneoscleral rims and cultured to confluence as approved by the Hines VA and Loyola University Chicago institutional review boards. SV40-transformed TM cells derived from a male glaucomatous patient (GTM3) and a male non-glaucomatous patient (NTM5) were a generous gift from Alcon laboratories. TM cell cultures were maintained at 37ºC in an atmosphere of 5% CO_2/95% air. At 90-100% confluence, cells were conditioned x24h in serum-free media and subsequently treated x24h with recombinant human TGF-β2 (5 ng/ml) as we have previously described.[10]

siRNA-Targeted Knockdown: TM cells were transfected with siRNA directed against a scrambled siRNA sequence (25-100 nM; negative control), Smad2 (25 nM), Smad3 (10 nM), or RhoA (100 nM) using Lipofectamine in a 1:1 mixture of OptiMEM and cell culture medium without serum or antibiotics/antimycotics. Primary TM cells were reverse-transfected for 8h, whereas GTM3 cells were transfected for 24h upon reaching confluence. Following transfection, culture media was replaced with serum-free medium, and cells were incubated in the absence or presence of TGF-β2 as indicated.

Western immunoblotting: Lysates from human TM cells treated as described above were prepared in 2X Laemmli's sample buffer and stored at -80ºC until use. Proteins (20-30 μg per lane) were resolved as we have previously described[15] and immunostained overnight at 4ºC in the presence of a 1:1,000 dilution of primary antibodies directed against: B2 receptor, phospho-Smad2 (Ser423/425), phospho-Smad3 (Ser465/467), total Smad2, total Smad3, RhoA (1:200 dilution) or GAPDH (1:10,000 dilution). Washed membranes were incubated for 1h at 23ºC in a 1:2500-1:10,000 dilution of peroxidase-conjugated secondary antibody. Immunostained proteins were visualized by enhanced chemiluminescence. Relative changes in protein content were quantified by densitometry and normalized to total GAPDH content as we have described previously.[16]

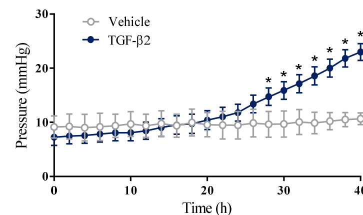
Real-Time RT-PCR: Total RNA was extracted from human TM cells using TRIzol reagent and reverse-transcribed as we have described previously.[10] Human-specific B2 mRNA or GAPDH cDNA sequences were amplified by real-time PCR on a Mini-Opticon PCR detection system. For each sample, the specificity of the real-time reaction product was determined by melt curve analysis. Relative fold-changes in B2 mRNA expression were normalized to GAPDH.

Porcine Anterior Segment Perfusion: Anterior segment perfusion are performed on-site at Hines VA Hospital using fresh porcine eyes obtained from a local abattoir. Globes are bisected aseptically at the equator, and the iris, lens, and vitreous gently removed to minimize pigment flocculation. The prepared anterior segment is mounted, cornea side up, to a sterile custom-made organ culture chamber. Mounted anterior segments are perfused at 37°C in a humidified tissue culture incubator under an atmosphere of 5% CO_2/95% air with pre-warmed DMEM supplemented with antibiotics and antimycotics. Porcine globes are continuously perfused at a constant flow rate of 4.5 μl/min and cultured for up to 4 additional days following pressure stabilization. IOP is monitored and recorded every 30 seconds (PowerLab 8/35 with LabChart Pro).

Statistical Analysis: Results are expressed as mean ± SD unless otherwise specified. Parametric data were analyzed by Student's *t-test* or by one-way ANOVA followed by either a Dunnett's or a Bonferroni's multiple comparison post-hoc analysis. In all cases, $p < 0.05$ was considered statistically significant.

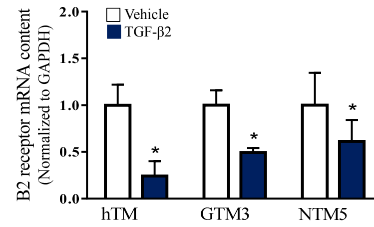
RESULTS

Figure 1. TGF-β2 increases IOP in cultured porcine anterior segments



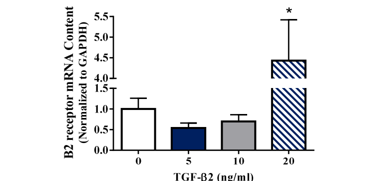
Pressures within porcine anterior segments were allowed to stabilize overnight prior to perfusion with Vehicle (400 nM HCl) or TGF-β2 (10 ng/ml) as indicated. Data shown as mean ± SEM (N=3) from a single experiment. $^{*}p < 0.05$, two-way ANOVA with Bonferroni's post-hoc analysis.

Figure 2. TGF-β2 attenuates constitutive B2 mRNA expression in human TM cells



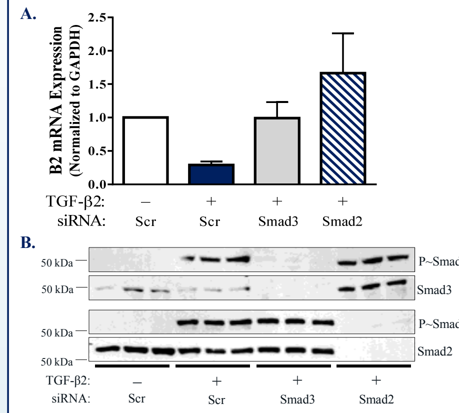
Primary (hTM) or transformed (GTM3, NTM5) human TM cells were conditioned in serum-free media x24h, and subsequently incubated x24h in the absence (200 nM HCl) or presence of TGF-β2 (5 ng/ml) as indicated. Relative content of B2 mRNA were quantified by qRT-PCR. Data shown are GAPDH-normalized fold changes from 2-3 separate experiments and expressed as the mean ± SD (N= 5-8). * $p < 0.05$; unpaired Student's *t-test*.

Figure 3. TGF-β2 dose-dependently alters constitutive B2 mRNA expression



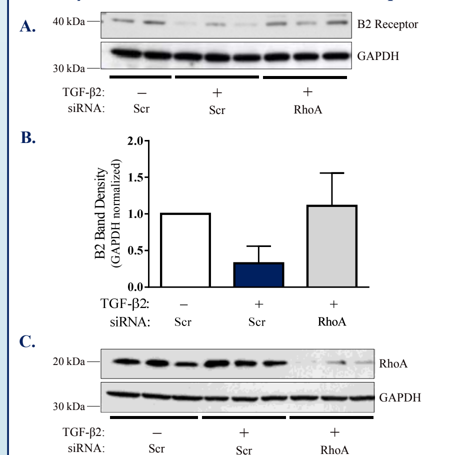
NTM5 cells were conditioned x24h in serum-free media and subsequently treated without (0) or with TGF-β2 (5-20 ng/ml) x24h as indicated. Relative changes in B2 mRNA content were quantified by qRT-PCR. Data are shown are the GAPDH-normalized fold changes from a single experiment and expressed as the mean ± SD (N=3). * $p < 0.05$; one-way ANOVA with Dunnett's post-hoc analysis.

Figure 4. Knockdown of receptor-associated Smads prevents TGF-β2 mediated decreases in B2 mRNA expression



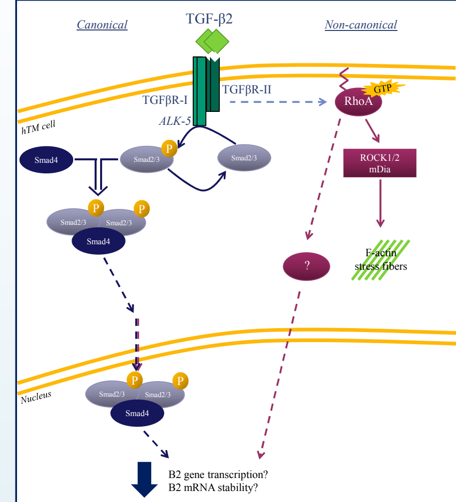
Primary hTM cells were reverse-transfected with scrambled (Scr, 25 nM), Smad3 (10 nM), or Smad2 (25 nM) siRNA as indicated. Transfected cells were subsequently incubated (24h) in the absence (200 nM HCL) or presence of TGF-β2 (5 ng/ml). **(A)** Relative content of B2 mRNA was quantified by qRT-PCR and normalized to GAPDH. Data shown are the mean ± SD from a single experiment (N= 2-3). **(B)** Immunoblot of phosphorylated and total Smad3 and Smad2 proteins. Results shown are from a single experiment, representative of 2-3 separate experiments.

Figure 5. Knockdown of RhoA prevents TGF-β2 mediated decreases in B2 mRNA expression



Transformed human GTM3 cells were transfected x24h with scrambled (Scr, 100 nM) or with RhoA (100 nM) siRNA as indicated, and incubated x24h in the absence (200 nM HCl) or presence of TGF-β2 (5 ng/ml). **(A)** Immunoblot of B2 receptor and GAPDH protein content. **(B)** Quantitative comparison of normalized B2 receptor protein content from immunoblot shown in **(A)**. **(C)** Immunoblot of RhoA and GAPDH proteins. Results are representative of 2 separate experiments.

PUTATIVE MECHANISM



CONCLUSION

Elevated content of TGF-β2 in the aqueous humor of POAG patients may increase IOP, in part, by attenuating constitutive B2 receptor expression within the conventional outflow pathway

REFERENCES

1. Quigley HA *et al.* The number of people with glaucoma worldwide in 2010 and 2020. *Br. J Ophthalmol* 2006; 90:262-267
2. Friedman DS *et al.* Prevalence of open-angle glaucoma among adults in the United States. *Arch Ophthalmol* 2004; 122:532-538
3. Leske MC. The epidemiology of open-angle glaucoma: a review. *Am J Epidemiol* 1983; 118: 166-191
4. Stubbs Jr. EB and Von Zee CL. Prenylation of Rho G-proteins: a novem mechanism regulating gene expression and protein stability in human trabecular meshwork cells. *Mol Neurobiol* 2012; 46:28-40
5. Tripathi, RC *et al.* Aqueous humor in glaucomatous eyes contains an increased level of TGF-beta2. *Exp Eye Res* 1994; 59: 723-727
6. Inatani M *et al.* Transforming growth factor-beta2 levels in aqueous humor of glaucomatous eyes. *Graefes Arch Clin Exp Ophthalmol* 2001; 239:109-113
7. Ochiai Y and OchiaiH. Higher concentration of transforming growth factor-beta in aqueous humor of glaucomatous eyes and diabetic eyes. *Jpn J Ophthalmol* 2002; 46:249-253
8. Gottanka J *et al.* Effects of TGF-beta2 in perfused human eyes. *Invest Ophthalmol Vis Sci* 2004; 45:153-158
9. Fleenor DL *et al.* TGF-beta2-induced changes in human trabecular meshworkL implications for intraocular pressure. *Invest Ophthalmol Vis Sci* 2006; 47:226-234
10. Von Zee CL *et al.* Transforming growth factor-beta2 induces synthesis and secretion of endothelin-1 in human trabecular meshwork cells. *Invest Ophthalmol Vis Sci* 2012; 53:5279-86
11. Webb JG *et al.* Bradykinin enhancement of PGE2 signaling in bovine trabecular meshwork cells. *Exp Eye Res* 2003; 76:283-289
12. Webb JG *et al.* Kinin modulation of conventional outflow facility in the bovine eye. *J Ocul Pharmacol Ther* 2006; 22:310-316
13. Fong CY *et al.* TGF-beta1 stimulates IL-8 release, COX-2 expression, and PGE(2) release in human airway smooth muscle cells. *Am J Physiol Lung Cell Mol Physiol* 2000; 279:L201-207
14. Tang SC *et al.* Bradykinin and high glucose promote renal tubular inflammation. *Nephrol Dial Transplant* 2010; 25:698-710
15. Von Zee CL *et al.* Increased RhoA and RhoB protein accumulation in cultured human trabecular meshwork cells by lovastatin. *Invest Ophthalmol Vis Sci* 2009; 50:2816-23.
16. Von Zee CL *et al.* Geranylgeranylation facilitates proteasomal degradation of Rho G-proteins in human trabecular meshwork cells. *Invest Ophthalmol Vis Sci* 2010; 52:1676-83

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