Mitochondrial-specific antioxidant XJB-5-131 attenuates endogenous TGF-β2 expression in human trabecular meshwork cells

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INTRODUCTION

Glaucoma is a leading cause of blindness, expected 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 currently have primary open angle glaucoma (POAG), the most prevalent form of the disease. Despite its overwhelming prevalence and socioeconomic impact, the treatment of patients with POAG remains restricted to non-specific interventions aimed at lowering intraocular pressure (IOP), a poorly-understood hallmark of POAG.

In healthy eyes, IOP is maintained through balanced production and outflow of aqueous humor (AH). In humans, more than 50% of AH leaves the eye through the conventional outflow system, including the trabecular meshwork (TM).[2] Under pathologic conditions, aberrant contraction of TM cells, or chronic accumulation of extracellular matrix (ECM) components, can increase resistance to AH outflow and promote elevated IOP.

Multiple studies have demonstrated that transforming growth factor (TGF)-β2, an anti-inflammatory cytokine that promotes ECM deposition and actin stress fiber organization in TM cells, is markedly elevated in the AH of patients with POAG.[3,4] *Despite this growing body of evidence supporting a causal role of TGF-β2 in the pathophysiology of POAG, the mechanisms underlying endogenous TGF-β2 expression and release remain unclear.*

Recently, production of reactive oxygen species (ROS) has also been linked with the development and progression of POAG.[5-10] In other cell types, elevated levels of ROS are known to promote production and release of TGF-β2.[11]

Here, we determined the effect of a novel mitochondrial-selective antioxidant (XJB-5-131) on production and release of TGF-β2 in cultured human TM cells and the porcine conventional outflow system.

METHODS

Porcine Anterior Segment Perfusion: Anterior segment perfusion experiments are performed using fresh porcine eyes obtained from a local abattoir. Globes are bisected aseptically at the equator, and the iris, lens, and vitreous gently removed. The prepared anterior segment is continuously perfused at a constant flow rate of 4.5 µl/min with pre-warmed DMEM supplemented with antibiotics and antimycotics. Anterior segments are cultured under a humidified atmosphere of 5% CO_2/95% air at 37°C and allowed to stabilize overnight. Following an initial 24h stabilization (washout) period, media perfusing porcine anterior segments was exchanged with DMEM containing either **(i)** vehicle (400 nM HCl) or recombinant active human TGF-β2 (10 ng/ml), **(ii)** vehicle (0.01% DMSO) or the TGFβRI/ALK-5 antagonist SB-431542 (10 µM), or **(iii)** vehicle (0.25% DMSO) or the proprietary mitochondria-targeted antioxidant XJB-5-131 (25 µM). IOP was monitored and recorded every 3 minutes (PowerLab 8/35 with LabChart Pro).

Human TM Cell Culture: An SV40-transformed human TM cell line (GTM3) derived from a male glaucomatous patient was a generous gift from Alcon Labs. Cell cultures were maintained at 37°C under an atmosphere of 5% CO_2/95% air. Human TM cells were cultured to confluence and treated x24h with vehicle (0.1% DMSO) or XJB-5-131 (10 µM).

Real-Time RT-PCR: Total RNA was extracted from human TM cells using TRIzol reagent, and 5 µg was reverse-transcribed using Super Script III First Strand Synthesis system. Human-specific TGF-β2 or GAPDH cDNA was amplified by qPCR on a Mini-Opticon PCR detection system. For each sample, the specificity of the reaction product was determined by melt curve analysis. The expression of GAPDH was unaltered by drug treatments; therefore, relative fold-changes in gene expression were normalized to GAPDH.

TGF-β2 ELISA: Levels of total TGF-β2 in cell culture media or effluent from porcine anterior segments were quantified using commercially-available ELISA kits (R&D Systems) according to manufacturer's instructions.

Statistical Analysis: Anterior segment perfusion results are expressed as the means ± SEM and analyzed by two-way ANOVA with a Bonferroni's multiple comparison post-hoc analysis. Cell culture data are expressed as the means ± SD or SEM and analyzed by Student's *t-test* or one-way ANOVA with a Bonferroni's multiple comparison post-hoc analysis. In all cases, statistical significance was defined as $p < 0.05$.

RESULTS

TGF-β2 signaling facilitates elevated IOP in cultured porcine anterior segments

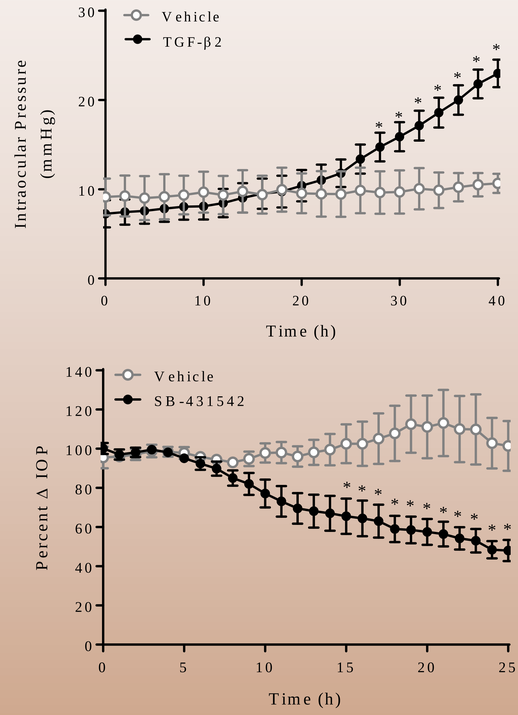

Figure 1. **(Top)** Stabilized porcine anterior segments (n=3) were perfused with either vehicle or TGF-β2, as indicated. Data shown are representative of two separate experiments. **(Bottom)** Stabilized porcine anterior segments (n = 5-6 per group) were perfused with either vehicle or SB-431542, as indicated. * $p < 0.05$; two-way ANOVA with Bonferroni's post-hoc analysis. (Pervan *et al*., under review.)

XJB-5-131 attenuates endogenous TGF-β2 mRNA expression

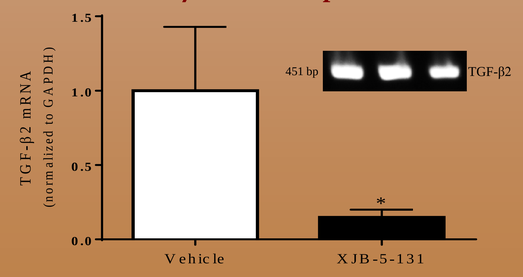

Figure 2. Content of TGF-β2 mRNA (n=6) in confluent transformed human TM cells incubated x24h in the absence (vehicle, 0.1% DMSO) or presence (10 µM) of XJB-5-131 as indicated. Data shown are the means ± SD. *, $p < 0.001$; unpaired Student's *t-test*. Inset: EtBr-stained agarose gel of TGF-β2 cDNA prepared from quiescent GTM3 cells (n=3).

Dose-dependent attenuation of endogenous TGF-β2 mRNA expression with XJB-5-131

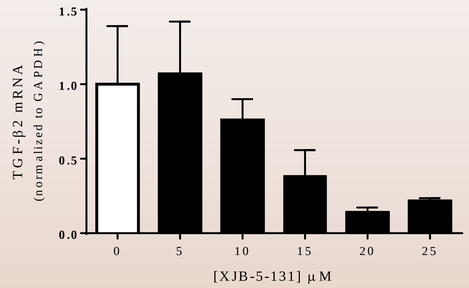

Figure 3. Content of TGF-β2 mRNA (n=3) in confluent transformed human TM cells incubated x24h in the absence (vehicle, 0.1% DMSO) or presence (5-25 µM) of XJB-5-131, as indicated. Data shown are the means ± SEM.

XJB-5-131 attenuates endogenous total TGF-β2 protein secretion

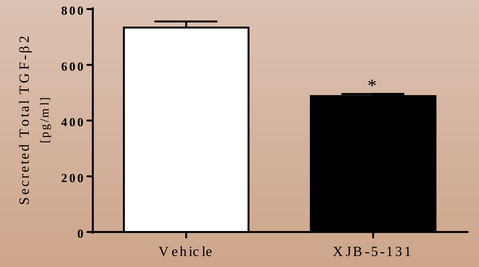

Figure 4. Absolute content of secreted total TGF-β2 protein (n=3) in GTM3 cells incubated x24h in the absence (vehicle, 0.1% DMSO) or presence (10 µM) of XJB-5-131 as indicated. *, $p < 0.001$, Student's *t-test*.

XJB-5-131 attenuates total TGF-β2 release from cultured porcine anterior segments

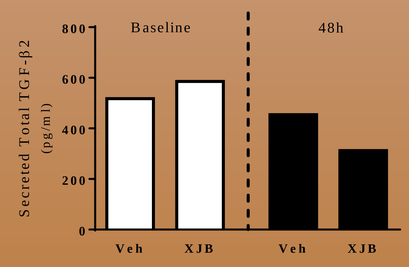

Figure 5. Stabilized porcine anterior segments (n=2 each) were perfused with media containing either vehicle (0.25% DMSO) or XJB (25 µM). Data shown are the average total TGF-β2 content in effluent collected at baseline (0h) and 48h post-treatment.

STRUCTURE

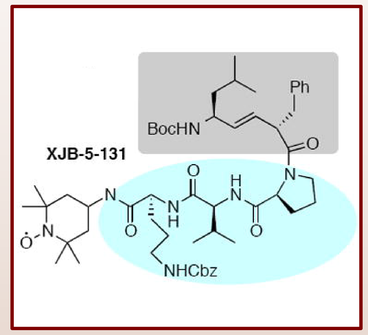

Figure 6. Chemical structure of XJB-5-131. From Skoda EM *et al.*, 2012.

XJB-5-131 *is a bi-functional antioxidant featuring a radical scavenger 4-hydroxy-2,2,6,6-tetramethyl piperidine-1-oxyl nitroxide conjugated to an alkene peptide isostere modification of the Leu-D-Phe-Pro-Val-Orn segment of the antibiotic gramicidin S that localizes to the mitochondrial membrane (Wipf P et al., 2005).*

SUMMARY/CONCLUSION

- Exogenous TGF-β2 promotes elevation of IOP
- Inhibiting endogenous TGF-β/ALK-5 signaling lowers IOP
- Human TM cells express and secrete TGF-β2
- XJB-5-131 dose dependently inhibits TGF-β2 mRNA expression
- XJB-5-131 inhibits secretion of TGF-β2 protein both *in vitro* and *ex vivo*

Mitochondrial-targeted antioxidant XJB-5-131 may lower IOP by attenuating constitutive TGF-β2 expression and secretion within the conventional outflow pathway

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