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Research article

Myocilin expression is regulated by retinoic acid in the trabecular meshwork-derived cellular environment

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ABSTRACT

Glaucoma is the leading cause of irreversible blindness and is usually classified as angle closure and open angle glaucoma (OAG). Primary open angle glaucoma represents the most frequent clinical presentation leading to ganglion cell death and optic nerve degeneration as a main consequence of an intraocular pressure' (IOP) increase. The mechanisms of this IOP increase in such pathology remain unclear but one protein called Myocilin could be a part of the puzzle in the trabecular meshwork (TM). Previously described to be transcriptionally regulated by glucocorticoids, the comprehension of the trabecular regulation of Myocilin' expression has only weakly progressed since 15 years. Due to the essential molecular and cellular implications of retinoids' pathway in eye development and physiology, we investigate the potential role of the retinoic acid in such regulation and expression. This study demonstrates that the global retinoids signaling machinery is present in immortalized TM cells and that Myocilin (*MYOC*) expression is upregulated by retinoic acid alone or combined with a glucocorticoid co-treatment. This regulation by retinoic acid acts through the *MYOC* promoter which contains a critical cluster of four retinoic acid responsive elements (RAREs), with the RARE-DR2 presenting the strongest effect and binding the RARα/RXRα heterodimer. All together, these results open up new perspectives for the molecular understanding glaucoma pathophysiology and provide further actionable clues on Myocilin gene regulation.

1. Introduction

The Myocilin (*MYOC*) gene was independently identified from forward genetic approaches and *in vitro* expression studies. Indeed, in early 1990[th], linkage study in a large family of juvenile-onset open-angle glaucoma (OAG) mapped a first locus designated *GLC1A* on the long arm of the chromosome 1 (Sheffield et al., 1993). Then, the genes of this locus were screened and pathogenic variants in the Myocilin gene were found to segregate with juvenile and adult-onset OAG (Stone et al., 1997). Otherwise, because *MYOC* expression was first isolated by differential library screening of trabecular meshwork (TM) cell cultures after glucocorticoids exposure, *MYOC* was transiently referred as *TIGR*, standing for Trabecular meshwork-Induced Glucocorticoid Response (Nguyen et al., 1998; Polansky et al., 1997). The Myocilin gene, *MYOC* (MIM: *601652), is expressed in most of the human eye compartments. The resulting 504 amino acid secreted glycoprotein is detected in cornea, trabecular meshwork, aqueous humor, iris, ciliary body, choroid, sclera, retina and the axons of optic nerve ganglion cells (Karali et al., 2000). Since pioneer induction studies in TM cells (for example see, Polansky et al., 1997; Kirstein et al., 2000), the understanding of the tissue specific regulation of *MYOC* expression has progressed. Steroids treatment is associated

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with increased levels of wild type Myocilin in trabecular meshwork cells, both *in vitro* and *in vivo* (Polansky et al., 1997; Clark et al., 2001; Ishibashi et al., 2002; Rozsa et al., 2006) whereas prostaglandin F2α decreases Myocilin expression (Lindsey et al., 2001). Interestingly, *MYOC* has been shown to be a delayed glucocorticoid-responsive gene in human trabecular meshwork cells (Shepard et al., 2001). Because of the cellular vitamin A final effectors (nuclear receptors belonging to the same superfamily than the glucocorticoids ones) are also upregulated by dexamethasone treatment (Ishibashi et al., 2002) and considering there absolute requirement for harmonious eye development as demonstrated by KO studies realized in mice (Kastner et al., 1994), they could be candidate actors of the delayed response. Retinoids signaling is mediated by the direct interactions of heterodimerized Retinoic Acid (RARs) and Retinoid X (RXR) nuclear receptors with specific consensus sequences (Retinoic Acid Responsive Elements; RAREs) in the promoter of a large set of genes. The vitamin A pathway displays pleiotropic functions such as cell differentiation, proliferation and apoptosis (Bastien and Rochette-Egly, 2004; Mark, 2007) that are essential for eye development and function (Cvekl and Wang, 2009; Samarawickrama et al., 2015). Together, these points lead us to focus on the interplay between the retinoids pathway and the Myocilin gene regulation in trabecular meshwork. Using a well-established TM derived cell line (TM5), we establish, that RAR/RXR heterodimers trigger specific RARE on *MYOC* promoter and that retinoic acid and dexamethasone have a synergistic effect on *MYOC* induction. To the best of our knowledge, this is the first study demonstrating that retinoic acid signaling is able to regulate directly *MYOC* expression. These findings open up new perspectives for the understanding of POAG pathophysiology and the testing of alternative therapeutic strategies in this pathology.

2. Material and methods

2.1. Bioinformatics analysis

MatInspector from Genomatix® program (http://www.genomatix.de) was used for *in silico* analysis to screen potential binding sites (RARE) for retinoic acid receptors. Briefly, the promoter sequence of the Myocilin gene was obtained from Ensembl (ENSG00000034971). The sequence comprised between nucleotides +1 and −4500 was submitted to Matinspector by defining Homo sapiens transcription factor binding sites and V$RXRF matrix. Matrix similarities with consensus binding site for RAR/RXR heterodimers were kept for the rest of the study.

2.2. Chemicals

Dimethyl sulfoxide (DMSO), *all-trans* retinoic acid (atRA) and dexamethasone were purchased from Sigma® (St Quentin Fallavier, France).

2.3. Cells culture

TM5 immortalized cell line was kindly provided by Alcon® research ltd (Fort Worth, TX, USA). TM5 cells were subcultured at 37 °C under 5% of CO2 in DMEM high glucose, with glutamax, with pyroxidine-HCL without sodium pyruvate supplemented with 2 mM of L-glutamine, 100 units of penicillin and 0.1 mg streptomycin (all products were purchased from Thermo-Fisher scientific (Saint-Aubin, France)).

2.4. RT-PCR and qPCR experiments

Total RNA was extracted from TM primary cells or TM5 cells using TRIZOL® after atRA (10^{-6}M) or DMSO treatment for 24, 48 or 72 h (Thermo-Fisher scientific (Saint-Aubin, France)). cDNA was synthetized using a superscript III first strand synthesis system for RT-PCR (Thermo-Fisher scientific (Saint-Aubin, France)). PCR experiments were done using specific oligonucleotides previously checked for their ability to amplify (Table 1). Results were analyzed on a 2% agarose gel. Quantitative PCR experiments (oligonucleotides described in Table 1) were done as previously described (Prat et al., 2012) on a Light Cycler 480® (Roche, Meylan, France) according to the MIQE guide-lines (Bustin et al., 2009) with the use of geometric mean of two housekeeping genes (36B4/RPLP0 and hRPS17). Results are expressed as mean of three independent experiments that were run in duplicate.

2.5. Western blot analysis

Protein extracts were obtained from TM primary cells or TM5 cells using RIPA buffer. Antibody against RARα, β, γ (1/200, sc-551, sc-552, sc-550, Santa-Cruz®, Heidelberg, Germany) and RXRα, β, γ (1/200, sc-553, sc-831, sc-555, Santa-Cruz®, Heidelberg, Germany) were used. For TM5 cells, this extraction was done after atRA (10^{-6}M) or DMSO treatment for 24, 48 or 72 h. Briefly, cell pellets were rinsed three times in PBS and 400 μL of RIPA were added. The mix was then placed on ice for 30 min with regular vortex mixing. Supernatants were collected and frozen at −80 °C. For western-blot, proteins were resolved on a SDS-PAGE gel electrophoresis (10%). Then, the transfer was done on nitrocellulose membrane (GE Healthcare®, Velizy, France) and saturated during 30 min with 5% skimmed milk in PBS-Tween (0, 1%). Antibody against Myocilin (1/200, sc20976, Santa-Cruz®, Heidelberg, Germany) or β-actin (1/1000, ab6276, Abcam®, Paris, France) were diluted in 5% skimmed milk in PBS-Tween (0, 1%) and incubated overnight at 4 °C. The membrane, rinsed three times with distillated water, was incubated at RT with a HRP coupled secondary antibody anti-rabbit (1/5000, ab6885, Abcam®) or anti-mouse (1/5000, ab6808, Abcam®) for one hour. The revelation was done using ECL plus Western-Blot kit (GE 164 healthcare®). Quantity One software (BioRad®) was used for quantification. Results are expressed as mean of three independent experiments that were run in duplicate.

2.6. Retinoid transcriptional machinery tests in TM primary cells

TM primary cells were transfected (using FugeneHD®, Roche, Meylan, France) with 1 μg DR5-tk-CAT (containing one copy of the retinoic acid-responsive element DR5 (direct repeat 5) upstream of the reporter gene CAT) and 0,15 μg of a CMV-β-Galactosidase vector serving as internal control to normalize variations in transfection efficiency. After a 24 h incubation, cells were rinsed and fresh medium containing the treatment was added (*i.e.*: retinol, atRA "retinol + ethanol", "retinol + bisdiamine", "retinol + ketoconazole") for 24 h. After washing the cells, CAT (Roche) and β-Galactosidase (Agilent technologies®, Les Ulis, France) assay were done on cell lysis. In all experiments, CAT activity was normalized by β-galactosidase activity according to both manufacturer protocols. Results are expressed as mean of three independent experiments that were run in duplicate.

2.7. Immunochemistry assays

TM5 cells were cultured in Lab-tek™ culture chambers (MC2, Clermont-Ferrand, France). After treatment, they were fixed in paraformaldehyde 4% in PBS at room temperature (RT) for 10 min and washed three times in PBS. After incubation in bovine serum albumin 3% for 30 min, primary antibody against Myocilin (1/200, sc20976, Santa-Cruz®) was then applied overnight à 4 °C. After